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(54) Title: MONOCLONAL ANTIBODY FRAGMENT TO HUMAN OVARIAN CANCERS

(57) Abstract

The biodistribution and pharmacokinetics of intact and F(ab')$_2$ fragments of radiolabeled (^{125}I) monoclonal antibody (Mab) MX-35 and a control mAB were compared in nude mice bearing xenografts of OVCAR-3 human epithelial ovarian cancer. The highest tumor to blood ratio was 12.8 for the F(ab')$_2$ fragment and 1.9 for intact Mab MX-35. Maximum tumor to normal tissue ratios were 64 for fragment and 12.3 for intact antibody. The maximum percent injected dose per gm (%IDg^{-1}) of Mab in tumor was 10.4 % for F(ab')$_2$ fragments and 1.6 % for intact antibody when administered intravenously and 8.2 % for F(ab')$_2$ fragments and 2.3 % for intact MX-35 when injected intraperitoneally. The finding of higher percentage ID g^{-1} for the fragments is unexpected and is in contrast to other published studies.

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MONOCLONAL ANTIBODY FRAGMENT TO HUMAN OVARIAN CANCERS

This invention was partially made with funds provided by the National Cancer Institute under grants CA-26184 and CA-08748. Accordingly, the United States Government has certain rights in this invention.

This invention relates to a method for the production of monoclonal antibodies (monoclonal antibodies) to restrictive antigenic human cell components especially in human ovarian tissues. Such monoclonal antibodies have use in cancer diagnosis and therapy, as well as other cell disorders.

Background

Conventional antisera, produced by immunizing animals with tumor cells or other antigens, contain a myriad of different antibodies differing in their specificity and properties. In 1975 Köhler and Milstein (Nature, 256:495) introduced a procedure which leads to the production of quantities of antibodies of precise and reproducible specificity. The Köhler-Milstein procedure involves the

fusion of spleen cells (from an immunized animal) with an immortal myeloma cell line. By antibody testing of the fused cells (hybridomas), clones of the hybridomas are selected that produce antibody of the desired specificity. Each clone continues to produce only that one antibody, monoclonal antibody (monoclonal antibody). As hybridoma cells can be cultured indefinitely (or stored frozen in liquid nitrogen), a constant, adequate supply of antibody with uniform characteristics is assured.

Antibodies are proteins that have the ability to combined with and recognize other molecules, known as antigens. Monoclonal antibodies are no different from other antibodies except that they are very uniform in their properties and recognize only one antigen or a portion of an antigen known as a determinant.

In the case of cells, the determinant recognized is an antigen on or in the cell which reacts with the antibody. It is through these cell antigens that a particular antibody recognizes, i.e. reacts with, a particular kind of cell. Thus the cell antigens are markers by which the cell is identified.

These antigenic markers may be used to observe the normal process of cell differentiation and to locate abnormalities within a given cell system. The process of differentiation is accompanied by changes in the cell surface antigenic phenotype, and antigens that distinguish cells belonging to distinct differentiation lineages or distinguish cells at different phases in the same differentiation lineage may be observed if the correct antibody is available.

The preparation of hybridoma cell lines can be successful or not depending on such experimental factors as nature of the innoculant, cell growth conditions, hybridization conditions etc. Thus it is not always possible to predict successful hybridoma preparation of one cell line although success may have been achieved with another cell line. But it is often true that selected monoclonal antibody may be representative of a class of monoclonal antibody raised by a particular immunogen. Members of that class share similar characteristics, reacting with the same cell antigen. Thus the invention includes hybridoma cell lines and monoclonal antibody with like or similar characteristics.

Progress in defining cell surface antigens is of great importance in differentiation and disease as markers for normal and diseased cells, thereby furthering diagnosis and treatment. Thus work on melanocytes was made possible by the recently discovered technique of culturing melanocytes from normal skin (Eisinger, et al., Proc. Nat'l. Acad. Sci. USA, 79 2018 (March 1982). This method provides a renewable source of proliferating cells for the analysis of melanocyte differentiation antigens. Likewise, a large number of cell lines derived from melanomas have now been established and these have facilitated the analysis of melanoma surface antigens. The advent of monoclonal antibodies has greatly accelerated knowledge about the surface antigens of malignant melanoma. cell markers on both melanomas and melanocytes have been identified. A panel of typing monoclonal antibodies has been selected which recognizes differentiation antigen characteristics at each stage of development in both melanocytes and melanomas. These differentiation antigens may be used to classify melanocytes and melanomas and to group them into characteristic sub-sets. [Dippold et al. Proc. Nat'l.Acad. Sci. U.S.A. 77, 6114 (1980) and Houghton, et al. J. Exp. Med. 156, 1755 (1982)]. Immunoassay of melanocytes and melanoma cells within sub-sets is thus made possible.

Initial recognition of differentiation antigens came about through analysis of surface antigens of T-cell leukemias of the mouse and the description of the TL, Thy-1, and Lyt series of antigens. (Old, Lloyd J., Cancer Research, 41, 361-375, February 1981) The analysis of these T-cell differentiation antigens was greatly simplified by the availability of normal T cells and B cells of mouse and man. (See Patents #4,361,549-559; #4,364,932-37 and #4,363,799 concerning monoclonal antibody to Human T-cell antigens).

The existence of human leukemia specific antigens has been suggested by studies using heterologous antibodies developed by immunization with human leukemic cells [Greaves, M.F. et al. Clin. Immunol. and Immunopathol 4:67, (1975); Minowada, J., et al. J. Nat'l. Cancer Insti. 60:1269, (1978); Tanigaki, N., et al. J. Immunol. 123:2906, (1979)] or by using autologous antisera obtained from patients with leukemia [Garret, T.J., et al., Proc. Nat'l. Acad. Sci. USA 74:4587, (1977); Naito, K., et al., Proc. Nat'l. Acad. Sci. USA, 80: 2341, (1983)]. The common acute lymphoblastic leukemia antigen (CALLA) which is present on leukemia cells from many patients with non-T, non-B, acute lymphoblastic leukemia (N-ALL), some chronic myelocytic leukemias (CML) in blast crisis and a few acute

T-lymphoblastic leukemias (T-ALL) was originally described using conventional rabbit heteroantisera [Greaves, M.F. et al. Supra].

By the autologous typing technique [Garret, T.J., et al. Supra; Naito, K., et al. Supra 1983; Old, L.J. Cancer Res. 41:361, (1981)], antibodies uniquely reacting with ALL cells were found in sera obtained from patients with ALL, and seemed to recognize very similar antigens to CALLA (Garret, T.J., et al. Supra; Naito, K., et al. Supra). Another leukemia associated antigen detected by heterologous antisera is the human thymus leukemia (TL)-like antigen, which is present on thymocytes as well as leukemia cells (Tanigaki, N. et al. Supra). This antigen, is therefore, a normal differentiation antigen which is composed of a heavy chain (MW 44,000-49,000) and light chain (MW 12,000-14,000) similar to the class I HLA antigens (Tanigaki, N., et al. Supra). These investigations have, however, been hampered by the need for vigorous absorptions with normal tissues as well as the relatively small quantity and low titer of the antisera.

In vitro production of monoclonal antibodies by the technique of Köhler and Milstein, Supra has provided a better system for the identification and detection of

leukemia specific antigens. A panel of monoclonal antibodies detecting cell surface antigens of human peripheral blood lymphocytes and their precursor cells have been investigated in detail [Reinherz, E.L., et al. Proc. Nat'l. Acad. Sci. USA 77:1588, (1980)]. While monoclonal antibodies detecting antigens characteristic for different lymphocyte lineages can be used for classification of human lymphocytic leukemia [Schroff, R.W., et al. Blood 59: 207, (1982)], such antibodies have only limited therapeutic applications. Monoclonal antibodies detecting human leukemia associated antigens have also been produced. These include several antibodies detecting the human equivalents of the murine TL antigens. One TL-like antigen is recognized by NA1/34 [McMichael, A.J., et al. Eur. J. Immunol. 9:205, (1979)], OKT6 (Reinherz, E.L., et al. Supra) and Leu 6 (R. Evans, personal communication). A second TL-like antigen is recognized by M241 (Knowles, R.W., et al. Eur. J. Immunol. 12: 676,1982). Monoclonal antibodies with specificities for common acute lymphoblastic leukemia antigens J-5 (Ritz, J., et al. Nature 283:583, 1980), NL-1 and NL-22 (Ueda, R., et al. Proc. Nat'l. Acad. Sci. USA 79:4386, 1982) have also been produced. Recently, Deng, C-T, et al. Lancet. i:10, 1982) reported a complement fixing monoclonal antibody (CALLA-2) which reacts with most cultured human T-ALL cell lines and also reacts with most fresh T-ALL cells.

Mouse monoclonal antibodies to human tumor cell surface antigens have been produced in many laboratories (Lloyd, K.O. (1983) In: Basic and Clinical Tumor Immunology, Vol. 1 (R.B. Herberman, Ed.), Nijhoff, The Hague (in press)). The intention of these studies often has been to identify tumor-associated antigens that could be useful in tumor therapy or diagnosis. An inherent difficulty in this approach is the diversity of antigens on the cell surface. Although it has been possible to identify some antigens with a very restricted distribution, antibodies to antigens that elicit very weak immune responses may be missed due to their scarcity. These restricted antigens may be quite difficult to identify. Also, immunization with a complex mixture of antigens such as tumor cells may suppress the antibody response to relatively less immunogenic molecules, in a manner resembling antigenic competition (Taussig, M.J. (1973). Curr. Top. Micro. Immuno. 60:125). Thus production of monoclonal antibody to restricted cell sites is an especially difficult task. The present invention provide cancer diagnosis and therapy and overcome problems heretofor encountered in the prior art with respect to ovarian and endometrial human cell antigens.

The production and characterization of mouse monoclonal antibodies (mAbs) to human tumor cells has recently been an active area of research in recent years. This effort was stimulated by many earlier immunological studies which suggested that existence of human tumor-specific antigens, but such evidence has not been conclusive, and has not been consistently reproduced in other laboratories, making the status of such antigens uncertain (reviewed in Old, L.J. Cancer Res. 41:361, 1981; Herberman, R.B. Cancer Res. 19:207, 1974; Kedar, E., et al. Adv. Cancer Res. 38:171 1983; North, R.J. Adv. Immunol. 35:89, 1984 and Weiss, P.W., Curr. Topics Micro. Immunol. 89:1,1980). Mouse monoclonal antibodies also have not yet provided conclusive identification of a human tumor-specific antigen, although a few possibilities have been reported (Schlom, J., et al. (1985) Adv. Cancer Res., 43:143-174; Tsuji, Y., et al. (1985) Cancer Res., 45:2358-2362; Tong, A.W., et al. (1984) Cancer Res., 44:4987-4992; Chin, J., et al. (1985) Cancer Res. 45:1723-1929). However, monoclonal antibodies to many new differentiation antigens have been obtained, and some of these have been recognized as having potential value in tumor diagnosis and therapy, particularly if they are expressed at higher levels in tumors than in normal cells. It should be considered that even monoclonal

antibodies reacting with numerous normal cell types are more specific than current therapeutic agents (drugs and radiation). In addition several antigens identified by monoclonal antibodies (all of which are mucin-like) appear to be valuable serum markers for particular cancer types (Herlyn, M., et al. (1982) J. Clin. Immunol., 2:135-140; Klug, T.L., et al. (1984) Cancer Res., 44:1048-1053; Lan, M.W., et al. (1985) Cancer Res., 45:305-310; Papsidero, L.D., et al. (1984) Cancer Res., 44:4643-4647, Hirota, M., et al. (1985) Cancer Res., 45:1901-1905).

Ovarian carcinoma is a promising target for monoclonal antibody therapy (as all cancers of other non-essential organs) in that tissue-specific differentation antigens are as useful as tumor-specific antigens. However, monoclonal antibodies specific for a particular epithelial cell type have been difficult to obtain. Of the large number of monoclonal antibodies obtained reacting with human carcinomas, only a few appear to be specific for a particular histological type, namely the prostate (Raynor, R.H., et al. (1984) J. Nat. Cancer Inst., 73:617-625; Frankel, A.E., et al. (1982) Proc. Natl. Acad. Sci. USA, 79:903-907), the lung (Stahel, R.A., et al. (1985) Int. J. Cancer 35:11-17) and the breast (Menard, S., et al. (1983) Cancer Res., 43:1295-1300) and the breast, and these specificities have not yet been independently confirmed.

A number of ovarian tumor antigens have been detected using xenogeneic polyclonal sera (reviewed in Lloyd, K.O. (1982) Serono Symposium No. 46 (M.I. Colnagki, G.L. Buraggi and M. Ghrone, Eds.) Academic press. N.Y. pp. 205-211) but none are related to the antigens of the invention. Other laboratories have also described monoclonal antibodies to human ovarian carcinoma different from those of the invention. Bhattacharya et al. (Bhattacharya, M., et al. (1982) Cancer Res., 42:1650-1654) produced an antibody to a saline-extracted antigen detected only in mucinous cyst adenocarcinomas of the ovary and in fetal intestine. Serous cyst adenocarcinomas, the most common ovarian carcinoma, did not contain this antigen. Bast et al. produced an antibody (OC 125) reactive with an antigen present on 6/6 ovarian carcinoma cell lines and one melanoma of 14 non-ovarian cell lines tested. This antibody reacted with sections of 12,20 ovarian carcinomas and was nonreactive with 12 non-ovarian carcinomas and with most normal tissues, including normal adult and fetal ovary. Weak reactivity was observed with adult fallopian tube, endometrium and endocervix (Bast, R.C., et al. (1981) J. Clin. Invest., 68:1331-1336; Kabawat, S.E., et al. (1983) Amer. J. Clin. Pathol., 79:98-104).

We herewith incorporate by reference our previous work in the field namely U.S. patent application S.N. 562,465, and Intern J. of Gynocol. Pathol. 4:121 (1985) concerning mAbs MH94, MF116, MD144, MH55, MF61, MH94 and MH99; also U.S. patent application 764,862 and J. Histochem. and Cytochem (1985) 33:1095-1102 concerning mAbs MU78, MT334 and MQ49 and U.S. patent application S.N. 556,579 and Hybridoma 2:pp 253-264 (1983) concerning mAb MH99.

Summary

Monoclonal antibody for ovarian cancers described. The antigenic profile of each of these monoclonal antibodies is presented with both serological and tissue reactivity studies in cancer and normal cell lines and tissue sections. These monoclonal antibodies form a panel useful for the diagnosis and therapy of ovarian cancers.

Ovarian epithelial cells form a simple cuboidal epithelium, and have no known function that distinguishes them from other epithelial cells; it is possible that there is no marker unique to these cells.

Also provided by this invention is an $F(ab')_2$ fragment of the monoclonal antibody MX35.

This invention also provides a method of detecting human ovarian cancer in a subject which comprises obtaining a suitable sample from the subject, contacting the suitable sample with an amount of the aforementioned $F(ab')_2$ MX35 fragment, labeled with a detectable marker, effective to and under conditions permitting the fragment to form a complex with an antigen present on human ovarian cancer cells if present in the sample, and detecting any complexes so formed, thereby detecting human ovarian cancer in the subject.

This invention further provides a method of treating human ovarian cancer in a subject which comprises administering to the subject an amount of the aforementioned MX35 fragment, conjugated to a therapeutic agent, such as a radioactive therapeutic agent, effective to treat human ovarian cancer.

This invention further provides a method of detecting human ovarian cancer in a subject which comprises administering to the subject an amount of the aforementioned MX35 fragment, labeled with a detectable marker, effective to and under conditions permitting the fragment to specifically form a complex with an antigen present on human ovarian cancer cells if present within the subject, and detecting the detectable marker labelling the antibody so complexed.

Detailed Description of the Invention

This invention provides an $F(ab')_2$ fragment of the monoclonal antibody MX35.

Also provided is the $F(ab')_2$ MX35 fragment labeled with a detectable marker. Detectable markers useful in the subject invention can readily be ascertained by those of ordinary skill in the art. Useful detectable marker include, but are not limited to, enzymes, for example alkaline phosphatase or horseradish peroxidase;

substrates for enzymes; compounds capable of fluorescing, for example fluorescein; and radioactive marker. Examples of radioactive markers useful for the subject invention include, but are not limited to, radioactive isotopes, for example radioactive iodine such as ^{131}I or ^{125}I, radioactive technetium, and radioactive indium, and compounds containing such radioactive isotopes. Other detectable markers may be found by those of ordinary skill, and such markers are useful for purposes of the subject invention.

This invention also provides the aforementioned $F(ab')_2$ MX35 fragment conjugated to a therapeutic agent. Therapeutic agents useful for the subject invention are those therapeutic agents which kill cancer cells, that is cells having a malignant phenotype. Therapeutic agents capable of killing cells having a malignant phenotype are well known to those of ordinary skill in the art, and any such therapeutic agent may be used in the subject invention. Useful therapeutic agents include drugs capable of killing malignant cells, for example doxorubicin; toxins; bacterial toxins, for example cholera toxin; and radioactive therapeutic agents, such as radioactive isotopes, for example ^{131}I, radioactive technetium, and radioactive indium, or compounds containing such radioactive isotopes.

This invention also provides a method of detecting human ovarian cancer in a subject which comprises obtaining a suitable sample from the subject, contacting the suitable sample with an amount of the aforementioned $F(ab')_2$ MX35 fragment, labeled with a detectable marker, effective to and under conditions permitting the fragment to form a complex with an antigen present on human ovarian cancer cells if present in the sample, and detecting any complexes so formed, thereby detecting human ovarian cancer in the subject.

Suitable samples for purposes of the subject invention include tissue samples, such as tissue biopsies. The tissue sample may be obtained from normal-appearing ovarian tissue. In one embodiment, the normal-appearing ovarian tissue sample is obtained from a

subject who may be predisposed to developing ovarian cancer, for example a subject whose family has a history of ovarian cancer. The tissue sample may, however, be obtained from abnormal-appearing tissue, for example from a growth or tumor. Such abnormal-appearing tissue may be abnormal-appearing ovarian tissue, but may also be abnormally-appearing tissue of any organ in the peritoneal cavity of a subject.

Another suitable sample is a fluid sample, such as, but not limited to blood, plasma, serum, mucus (for example cervical mucus), fluid obtained from the peritoneal cavity, including ascites fluid (i.e. fluid which has collected in the peritoneal cavity of a subject as a result of infection occurring, for example, from a growth or tumor).

Other suitable samples useful for the subject invention may be ascertained by those of ordinary skill in the art.

Complexes are detected by detecting the detectable marker labelling the antibody fragment, and the means of detection will depend on the particular detectable marker chosen for use in the subject invention. For example, if the detectable marker is an enzyme, antibody fragment-antigen complexes may be detected by contacting the sample with a substrate for the enzyme and monitoring production of the enzyme product. As another example, if the detectable marker is a radioactive marker, the antibody fragment-antigen complexes may be detected by X-ray, by counting radioactive emissions, or by scintillation counting. An appropriate detection means may be ascertained by those of ordinary skill in the art.

In one embodiment, detecting human ovarian cancer in the subject comprises detecting a micrometastatic tumor or micrometastatic tumors in the suitable sample obtained from the subject. A micrometastatic tumor is a small tumor which has metastasized from an ovarian cancer tumor. In one embodiment, a micrometastatic tumor is less than about 1.5 cm in diameter. In another embodiment

a micrometastatic tumor is less than about 1 cm in diameter. In a further, a micrometastatic tumor is less than about 5mm in diameter, and in another embodiment a micrometastatic tumor is less than about 1mm in diameter.

In another embodiment, detecting human ovarian cancer in the subject comprises detecting an epithelial ovarian carcinoma or epithelial ovarian carcinomas in the subject.

This invention further provides a method of treating human ovarian cancer in a subject which comprises administering to the subject an amount of the aforementioned MX35 fragment, conjugated to a therapeutic agent, such as a radioactive therapeutic agent, effective to treat human ovarian cancer.

For purposes of the subject invention, "treating human ovarian cancer" means killing ovarian cancer cells. Note that the ovarian cancer cells are not limited in location in the ovaries, but may comprise metastases or may be shed ovarian cancer cells located in other parts of the subject's body, for example in the peritoneal cavity of the subject.

Suitable modes of administration of antibody fragments are known in the art, and such method may be used in the invented treatment method. Examples of suitable forms of administration include intravenous injection and intraperitoneal injection. Administration may comprise administering the subject antibody fragment into the subject's peritoneal cavity.

The effective amount may be determined by methods known in the art. Typically, the subject is given a small dose of the antibody fragment conjugated to the therapeutic agent, and the dosage is increased until the subject cannot tolerate adverse side effects caused by the antibody fragment-conjugated therapeutic agent.

In one embodiment of the aforementioned method of treatment, the

human ovarian cancer cells comprise a micrometastatic tumor or micrometastatic tumors. Micrometastatic tumors are described above.

This invention further provides a method of detecting human ovarian cancer in a subject which comprises administering to the subject an amount of the aforementioned MX35 fragment, labeled with a detectable marker, effective to and under conditions permitting the fragment to specifically form a complex with an antigen present on human ovarian cancer cells if present within the subject, and detecting the detectable marker labelling the antibody so complexed.

Appropriate methods of detection, as described above, depend on the chosen detectable marker. As described above, an appropriate method of detection can be determined by one of ordinary skill in the art.

In one embodiment of the above-described invented method of detecting human ovarian cancer in a subject, detecting human ovarian cancer in the subject comprises detecting a micrometastatic tumor or micrometastatic tumors in the subject. Micrometastatic tumors are as described above. In a further embodiment, the micrometastatic tumor or micrometastatic tumors are located in the subject's peritoneal cavity.

Suitable modes of administration include intravenous injection and intraperitoneal injection. Administration of the antibody fragment labelled with the detectable marker may comprise administering the fragment into the subject's peritoneal cavity.

This invention will be better understood from the Examples in the experiments which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of, and are not intended to, nor should they be construed to, limit the invention as described more fully

in the claims which follow thereafter.

We have investigated monoclonal antibodies to ovarian carcinomas for 5 years in an attempt to identify tumor-specific or tissue-specific markers (Mattes, M.J., et al. (1984) Proc. Nat'l. Acad. Sci. USA, 81:568-572; Cordon-Cardo, C., et al. (1985) Int. J. of Gynocol. Pathol., 4:121-130; Mattes, M.J., et al. (1985) J. Histochem. Cytochem., 33:1095-1102). Initially cell lines were used for immunization and screening. Since ovarian carcinoma cell lines are very rare, and are likely to be nonrepresentative of tumors occurring in vivo, we began another study in which we immunized mice with fresh tumor specimens, and used frozen tissue specimens as targets for screening. Here we describe 3 mAbs produced in this way, and a fourth, previously undescribed, produced earlier by immunization with an ovarian carcinoma cell line. These 4 monoclonal antibodies react with most of all fresh ovarian carcinomas and with a distinct range of normal epithelial cells. We describe their reactivity with fresh ascites carcinoma cells, and their lack of reactivity with normal mesothelium; these properties show a potential use in the effective intraperitoneal immunotherapy. We here describe the reactivity of these monoclonal antibodies on a large panel of normal and malignant cell lines and on frozen sections of normal human tissues, as well as some biochemical characteristics of the antigens recognized.

Target cells. The origin and culture of cell lines derived form human tumors, normal human fibroblasts and normal kidney epithelial cells have been described (Mattes, M.J., et al. Proc. Natl. Acad. Sci. USA (1984) Supra). A summary of the cell lines used is given in Table I. Normal tissues were obtained at surgery or autopsy. The ovarian cyst used for screening hybridoma supernants was a serious cystadenoma. Ovarian adenocarcinomas tested included serous (7 specimens), endometrioid (2) and mucinous (1), tumor cell liver was obtained from human cancer serology laboratory and further tissue specimens from Dr. C. Cordon-Cardo and Dr. Virginia K. Pierce at SKI. Tissues were covered with O.C.T. Compound (Miles laboratories, Elkhart, IN) and frozen in a slurry of 2-methyl-butane cooled in liquid nitrogen.

Production of mouse monoclonal antibodies. The monoclonal antibodies described were obtained from 3 fusions. For the generation of MT179, (Balb/c X C57BL/6)F1 mice were immunized the the ovarian carcinoma cell line SK-OV-4. Intraperitoneal injections of approximately 0.1 ml of packed cells were given twice at an interval of two weeks. The other monoclonal antibodies were obtained after immunizing mice with a mixture of 4 fresh ovarian carcinoma specimens, including 2 samples of ascites cells and 2 solid

tumors, using approximately 10^7 x cells of each specimen. The mixture was suspended in 1.0 ml and injected i.p. three times at 3 week intervals. To immunize with solid tumor specimens, fragments of frozen tumor were thawed, placed in approximately 2 volumes of Dulbecco's phosphate-buffered saline (DPBS[3], Gibco, Grand Island, NY), teased with scalpels and pressed through a fine still screen. This preparation was stored frozen for subsequent injections. Ascites cells were prepared as described in the accompanying paper; after thawing, they were washed once with DPBS. Three days after the last injection, the fusion of immune spleen cells with mouse myeloma MOPC-21 NS/1 cells was performed as described (Mattes, M.J., et al. (1983) Hybridoma 2:253-264). Initially cells were plated in 480 wells (Costar #3524, 24-well plates). Hybridoma cultures were subcloned at least twice by limiting dilution in 96-well plates on a feeder layer of normal mouse spleen cells. For mAb at MT179, culture supernatants were tested for antibody activity on a panel of cultured cells consisting of immunizing cell line and other types of human tumor cells. For the other monoclonal antibodies, supernatants were tested for reactivity on cryostate sections of a benign ovarian cyst. The specificity of epithelial-specific supernatants was tested further on various frozen sections, cell lines, and ABO blood

group-related antigen preparations, as described below. Since several months were required for this specificity analysis, cells from all original wells were frozen in 10% dimethyl sulfoxide, using 2 vials for each well, just after the original supernatants were collected. For cloning, cells were thawed, grown briefly in a well of a 24-well plate, re-tested for antibody activity and cloned using our usual procedure above. Cloned hybridoma cells were injected subcutaneously into nu/nu mice. Sera from mice with progressively growing tumors were collected and used for serological and biochemical characterization. Antibody subclass was determined by immunodiffusion in agar with anti-Ig heavy chain-specific reagents (Bionetics, Kensington, MD).

Serological procedures. Red cell rosetting methods for adherent cultured for 1-4 days and nonadherent target cells were carried out as described previously (Farr, A.G., et al. (1981) J. Immunol. Methods 47:129-144; Graham, R.C., et al. (1965) J. Histochem. Cytochem. 13:150-152). The immune Rosetting assay was done as described (Carey, T.E., et al. (1976) Proc. Natl. Acad. Sci. USA 73:3278. For adherent target cells, 200-500 trypsinized cells were plated in 0.01 ml in wells of Terasaki plates (Falcon Microtest plates 3034) and cultured for 1-4 days. Nonadherent target

cells were attached to the wells by pretreating the wells for 45 min. at room temperature with Conconavalin A (Con A, grade IV, Sigma Chemicals, St. Louis Mo) at 1.0 mg/ml in DPBS. After washing the plates twice and blotting, target cells in DPBS were added and incubated for 45 min at room temperature Mattes, M.J. et al. J. Immunol. Meth 61:145 (1983). To test for neuraminidase sensitivity, target cells were treated for 1 hr at 37 with Vibrio cholerae neuraminidase (Calbiochem-Behring, La Jolla, CA) diluted 1:10 in 0.05 M citrate buffer pH 5.5, 0.1 M NaCl, 0.01 M CaC12.

Cytoplasmic antigens were detected using an immunoperoxidase method as described (Farr, A.G., et al. (1981) J. Immunol. Meth. 47:129; Graham, R.C. Jr., et al. (1965) J. Histochem. Cytochem. 13:150-152) washed twice with PBS, then fixed with 2.0% buffered formaldehyde (Farr, A.G., et al. (1981) J. Immunol. Methods 47:129-144) for 30 min. All incubations were at room temperature. After 2 washes with PBS, they were incubated with 0.05% NP40 in PBS for 15 min. After 2 washes with PBS, 5% fetal bovine serum (FBS), monoclonal antibody was added, starting with a 1:500 dilution. After a 45 min incubation, plates were washed twice and peroxidase-conjugated rabbit anti-mouse Ig (DAKO P161, Accurate Chemicals, Westbury NY) was added (prepared immediately before use by mixing 1.0 ml 0.05 M acetate buffer pH 5.0, 0.05 ml 3-amino-9-ethyl carbazole at 4.0

mg/ml in N,N-dimethylformamide, and 0.005 ml 3.0% hydrogen peroxide (Graham, R.C. Jr., et al. (1965) J. Histochem. Cytochem. 13:150-152). After 15 min, the plates were washed twice with PBS, once with water, and examined.

Immunoperoxidase staining of tissue sections using the ACC method was also carried out as described previously (Mattes, M.J., et al. (1985) J. Histochem. Cytochem. 33:1095-1102).

Procedures for absorption of Ab activity, using cells scraped from culture flasks, have been described [Hirota, M., et al. (1985) Cancer Res. 45:1901-1905]. To test heat stability of antigens, cells were heated to 100°C for 5 min, then washed once before use in absorptions. Blood leukocytes and erythrocytes were tested by immunofluorescence as described (Mattes, M.J., et al. (1984) Proc. Natl. Acad. Sci. USA 81:568-572), using the monoclonal antibody at 1/50 and fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) at 1/40. Reactivity with blood group A, B, H, Lewis^a, Lewis^b, X and Y determinants (Lloyd, K.O., et al. (1968) Proc. Natl. Acad. Sci. USA 61:1470-1477) was determined by a solid phase enzyme-linked immunoassay as described (Lloyd, K.O., et al. (1983) Immunogenetics 17:537-541), except that the antigen preparations were dissolved in water.

Biochemical analysis. Each antibody was tested for its ability to precipitate an antigen from the spent medium and from detergent-solubilized cell extracts after labeling by 3 methods: metabolic incorporation of [^{3}H]glucosamine (Mattes, M.J., et al. (1984) Proc. Natl. Acad. Sci. USA, 81:568-572), metabolic incorporation of [^{35}S]methionine (Mattes, M.J., et al. (1984) Supra) or chloramine T ^{125}I labeling of solubilized cell membranes (Mattes, M.J., et al. (1983) Hybridoma 2:253-264). NP40 solubilization of labeled cells (Mattes, M.J., et al. (1983) Supra) and immunoprecipitation procedures (Mattes, M.J., et al. (1984) Supra) have been described previously. To test heat stability, radio labeled extracts were heated at 100 for 5 min; precipitated proteins were removed by centrifugation (7,000 rpm, 15 min), then standard immunoprecipitations were performed. Preparation of chloroform; methanol, 2:1 cell extracts and their use in inhibitions assays has been described (Mattes, M.J., et al. (1984) Supra).

The examples below are for illustrative of the invention without limiting it.

Example I

Of the 4 monoclonal antibodies described here, one, MT179, was produced by immunization and screening with an ovarian carcinoma cell line, SK-OV-4. The assay employed was immunoperoxidase staining of fixed and permeabilized cells, which was intended to detect primarily cytoplasmic antigens. Initial selection of hybridomas to be cloned was based on nonreactivity with a panel of melanomas and astrocytomas, so we expected that monoclonal antibodies restricted to epithelial differentiation antigens would be selected. Other monoclonal antibodies produced from the same fusion were previously described (Mattes, M.J., et al. (1985) J. Histochem. Cytochem., 33:1095-1102). Three other monoclonal antibodies were obtained by immunization and screening with fresh tissue specimens. Immunization was with a mixture of 4 fresh ovarian carcinomas, as described. The mAbs were screened on cryostat sections of a benign serous ovarian cyst. This cyst specimen was chosen because the lining epithelial cells appeared morphologically similar to the simple cuboidal epithelial cells of the normal ovary, but were preserved better than normal ovarian epithelial cells in frozen sections. Therefore, this screening was primarily designed to detect tissue-specific epithelial differentiation antigens. Initial selection of hybridomas

was on the basis of reactivity with epithelial cells and non-reactivity with connective tissue and blood vessels in the sections. Of 393 supernatants tested, 16 were epithelial-specific. A much larger number, roughly 1/3 of the total supernatants, showed reactivity with all cells in the section. Before subcloning, we performed additional tests of specificity, in an effort to select ovarian-specific monoclonal antibodies. The 16 supernatants were tested on sections of normal colon and the 5 supernatants, which were negative were retained, these supernatants were also negative on normal skin. Since many epithelial differentiation antigens have been identified as ABO blood group-related antigens, we tested these five supernatants on a panel of mucins containing the antigens A, B, O, Le^a, Le^b, X and Y. Two of the supernatants reacted with the A antigen. The remaining 3 supernatants, designated MW162, MW207 and MX35, were then tested against cryostat sections of 5 solid ovarian tumors; all 3 reacted with 5/5 specimens. They were then tested on a panel of 30 cell lines, including ovarian carcinomas, other carcinomas and other tumor types. This was done partially to confirm the specificity of the antibodies, but also to select positive cell lines which could then be used as targets for screening subclones. Target cells were tested by 2 assays: a rosetting assay to detect cell surface antigens and an

immunoperoxidase assay on fixed, permeabilized cells to detect primarily cytoplasmic antigens. All three supernatant antibodies reacted with some ovarian carcinoma cell lines and were negative on melanoma and astrocytoma cell lines; they also reacted with some non-ovarian carcinoma cell lines. Frozen cells from these three original wells were thawed, established in culture, tested for retention of reactivity, then cloned twice by limiting dilution before expansion.

The four monoclonal antibodies described were all non-reactive with blood leukocytes and erythrocytes by immunofluorescence. Also, they did not react with any of the ABO blood group-related antigen preparations tested.

MT179. Ab MT179 is an IgG1. Although it reacts strongly in immunoperoxidase assays, it has not precipitated a detectable component from ovarian carcinoma SK-OV-4 or colon carcinoma SW 480 cells labeled with [^{35}S]methionine, [^{3}H]glucosamine of ^{125}I. In absorption experiments, the antigen was destroyed by heating at 100° for 5 min, suggesting that it is a protein. MT179 was detected initially by immunoperoxidase staining of fixed, permeabilized tissue culture cells, which produced cytoplasmic staining, but MT179 was also detected on the cell surface of SK-OV-4 by a rosetting assay.

In frozen sections, MT179 was detected in a number of normal epithelial cells, namely in the colon, lung, skin, pancreas and breast (Table 1). Other epithelial cells and all non-epithelial cells were negative. MT179 was detected also in sections of 8/10 ovarian carcinomas. Expression in 147 tissue culture cells lines is summarized in Table 2. MT179 was detected by immunoperoxidase staining in 3/8 ovarian carcinomas (SK-OV-4, SW626, A7), 2/2 uterine carcinomas (SK-UT-2, ME-180), 9/11 colon carcinomas (SW480, SW620, SW1116, SW1222, SW1417, SK-CO-1, -13, CaCO-2, 4J), 5/9 bladder carcinomas (Scaber, RT4, 5637, JON, SW780), 3/4 pancreatic carcinomas (CAPAN-1, -2, A3), 8/11 lung carcinomas (SK-LC-2, -3, -4, -5, -7, -9, -11, -12) 2/5 breast carcinomas (MCF-7, SK-BR-7), 2/17 renal carcinomas (SK-RC-17, -35), 1/2 prostate carcinomas (DU145), 1/1 bile duct carcinomas (Charles) and 1/1 choriocarcinoma (SVCC), with a reciprocal titer ranging from 500-8,000. It was negative on 21 melanomas, 16/17 astrocytomas, 5 neuroblastomas and 26 hematopoietic tumors of various types. Regarding cultured normal cells, 3 fibroblast cultures were negative and 2/3 kidney epithelial cultures were positive.

MW162. MW162 is an IgM. It precipitated an antigen from [^{3}H]glucosamine labeled SK-OV-6 that migranted at the top of a 9% acrylamide gel (greater than 300,000

daltons). The antigen could still be precipitated after incubating the labeled extract at 100 for 5 min. These characteristics suggest that the antigen is a carbohydrate determinant on a mucin or proteoglycan. The antigen was not detectably precipitated from cell extracts labeled with [^{35}S]methionine or ^{125}I. Reactivity of MW162 with SK-OV-4 was not affected by pretreatment of the cells, after permeabilization, with neuraminidase. The antigen was not detected, by inhibition, in a glycolipid fractions of SK-OV-6 prepared by chloroform: methanol extraction.

In frozen sections, MW162 was detected in many epithelial cells, namely in the esophagus, stomach, bronchus, lung, kidney distal tubules, pancreas, thyroid, uterus and breast (Table 2. Other epithelial cells, such as in the colon and skin, were negative, as were all non-epithelial cells examined. The antigen was detected in frozen sections of 10/10 fresh ovarian carcinomas. Often staining in sections was concentrated at the luminal edge of cells.

Its distribution in 105 tissue culture cells is shown in Table 2. It was detected most readily in the cytoplasm by immunoperoxidase staining, so this assay was used for screening tissue culture cells; the antigen was

detected weakly and inconsistently on the cell surface by rosetting. It was detected in 5/8 ovarian carcinomas (SK-OV-3, -4, -6, Colo 316, A10), 2/9 colon carcinomas (SW1116, SW1222), 3/6 bladder carcinomas (JON, VM-CUB-1, -2), 2/3 pancreatic carcinomas (CAPAN-1, -2), 6/8 lung carcinomas (SK-LC-1, -3, -7, -8, -17, -21), 4/4 breast carcinomas (MCF-7, SK-BR-5, -7, CAMA), 2/11 renal carcinomas (SK-RC-7, -18) and 1/1 choriocarcinoma (SVCC), with a reciprocal titer ranging from 500-2,000. It was negative on 10 melanomas, 10 astrocytomas, 4 sarcomas and 20 hematopoietic tumors of various types. Regarding normal cultured cells, it was negative on 4 fibroblast cultures and positive on 3/3 kidney epithelial cultures.

MW207. MW207 is an IgG1. Of the antigens described here, it is the only one that was recognized initially as a cell surface antigen, so the rosetting assay, rather than the immunoperoxidase assay, was used to screen cell lines. It has not precipitated a detectable component from ovarian carcinoma SK-OV-6 or renal carcinoma SK-RC-18 labeled by any of the 3 isotopes described in Materials and Methods. In absorption experiments, it was destroyed by heating to 100 for 5 min, suggesting that the determinant recognized is a protein. [Three ml of packed cells absorbed all detectable Ab activity, while 10 times more heat-treated cells had no absorption activity.]

In frozen sections, MW207 was detected on certain epithelial cells only, namely in the bronchus, lung, kidney proximal tubules, pancreas thyroid, uterus and breast (Table 1. In the pancreas, only the cells lining ducts were stained (Fig. 2). MW 207 was also detected in sections of 10/10 ovarian carcinomas. On 103 cell lines (Table 2) MW207 was present on 5/8 ovarian carcinomas (SW626, A7, A10, SK-OV-3, -6), 5/9 colon carcinomas (SW620, SW837, SW116, SW1222, SK-CO-10), 1/6 bladder carcinomas (VM-CUB-1), 2/3 pancreatic carcinomas (CAPAN-2, ASPC-1), 6/8 lung carcinomas (SK-LC-1,-7,-8,-14,-21,LcLL), 3/4 breast carcinomas (MCF-7, SK-BR-5, -7), 10/11 renal carcinomas (SK-RC-1, -7, -10, -15, -18, -29, -33, -42, -45, Caki 1), 1/1 teratocarcinomas (Tera-1) and 1/1 choriocarcinoma (SVCC) with reciprocal titers ranging from 10^3 to 10^5. The most strongly reactive target cells included carcinomas of the ovary, colon, bladder, lung, breast and kidney. MW207 was negative on 10 melanomas and 18 hematopoietic tumors, but reacted weakly with 2/10 astrocytomas and 1/4 sarcomas. Hence this Ab appears to be not strictly restricted to epithelial cells, although in frozen sections only epithelial cells were detectably stained. Regarding normal cells, MW207 was negative on 4 fibroblast cultures and positive on 2/3 kidney epithelial cell cultures.

MX35. MX35 is an IgG1. Although it reacted strongly in immunoperoxidase assays, it has not precipitated a detectable component from ovarian carcinoma SK-OV-6 or renal carcinoma SK-RC-18 cells, labeled by any of the 3 isoptopes described above. Heat stability could not be determined by absorption experiments, since the AB activity, diluted to near the endpoint, was not absorbed by an equal volume of unheated packed A10 cells, due perhaps to a low level of antigen exposure in scraped cells. MX35 was initially recognized as a cytoplasmic antigen, but was also detected by rosetting on the cell surface of the ovarian carcinoma line A10.

In frozen sections, MX35 was detected in epithelial cells of the normal bronchus, lung, kidney collecting ducts, thyroid and uterus (Table 1). All other tissues examined were negative. MX35 was also detected in sections of 10/10 fresh ovarian carcinomas. Staining was often concentrated at the luminal edge of cells. In tissue culture cell lines, MX35 expression was rare (Table 2), being detected on only 3/8 ovarian carcinomas (A7, A10, SK-OV-6), 1/8 lung carcinomas (SK-LC-1) and 3/11 renal carcinomas (SK-RC-18), -33, -53), with reciprocal titer of 500-32,000. The most strongly positive cell lines were SK-LC-1, SK-RC-18 and SK-RC-53. Since, as noted above,

10/10 fresh ovarian carcinomas were positive, the data suggests that this antigen may be lost during adaptation of tumor cells to tissue culture. Regarding normal cells, MX35 was negative on 4 fibroblast cultures and positive on 1/3 kidney epithelial cell cultures. Considering the results with both normal tissues and cell lines, MX35 is the most restricted of the antigens described here.

We have described above 4 distinct epithelial differentiation antigens identified by monoclonal antibodies. From their distribution on normal tissues and cell lines, it is clear that the 4 antigens are different from each other. We are not aware of antigens described by other investigators that are likely to be identical to these. Three of the monoclonal antibodies did not precipitate a detectable radio-labeled component, so little is known about the biochemical nature of the antigens recognized. The reason for the lack of immmunoprecipitation is not known, but possibilities include the following: 1) The antigen recognized is a minor cell constituent with a slow turn-over, so is not labeled adequately. 2) The antigen lacks glucosamine, methionine and tyrosine. 3) The antigen is either not extracted or denatured by the detergents used to solubilize the cells. MT179 and MW207 were heat labile, suggesting that they are proteins.

MAbs to differentiation antigens have a number of possible applications in cancer diagnosis and therapy as well as in more basic studies for cell biology and differentiation. In the example, we describe results of immunofluorescent staining of fresh ovarian carcinoma ascites cells, using these 4 monoclonal antibodies, which suggest that they are potentially useful in intraperitoneal therapy of such tumors. In addition, these Abs would probably be helpful in identifying rare carcinoma cells in peritoneal washings or lymph nodes, as has been described with other mABs (Ghosh, A.K., et al. (1983) J. Clin. Pathol. 36:1150-1153; and Johnston, W.W., et al. (1985) Cancer Res., 45:1894-1900). Also, a significant number of intraperitoneal carcinomas derive from an unknown or uncertain primary. These monoclonal antibodies can be helpful in determining the origin of the primary, but such an application would require prior extensive tests with frozen sections of tumors of known histological types. MX35 seems most useful in this regard, since it is the most restricted of the antigens described here, being negative on carcinomas of the colon, bladder, pancreas and breast, but positive on a proportion of carcinomas of the ovary, lung and kidney. Sections of 10/10 ovarian carcinomas (the only type of carcinoma tested) were positive. Although many normal epithelial cells express these antigens, there may be

certain tissue types in which the antigens are markers of malignancy. For example, MW162 and MW207 were negative on the normal colon, but were positive on some colon carcinoma cell lines. Further studies on cryostat sections of carcinomas of various types are required to investigate this possibility. Also, considering that most cancer serum markers defined by monoclonal antibodies have been characterized as mucins (Herlyn, M., et al. (1982) J. Clin. Immunol. 2:135-140; Klung, T.L., et al. (1984) Cancer Res., 44:1048-1053; Lan, M.S., et al. (1985) Cancer Res., 45:305-310; Papsidero, L.D., et al. (1984) Cancer Res., 44: 4653-4647; Hirota, M., et al. (1985) Cancer Res., 45:1901-1905), the mucin-like antigen identified by MW162 should be a potential serum marker.

We have not yet obtained monoclonal antibodies to tumor-specific or tissue-specific antigens, and this negative result warrants some discussion. Other laboratories have had similar results, although one report of ovarian carcinomas tumor-specific monoclonal antibodies has recently appeared (Tsuji, Y., et al. (1985) Cancer Res., 45:2358-2362). We have performed 22 fusions after immunizing mice with various ovarian carcinoma cell lines. From these fusions, monoclonal antibodies that are tumor-or tissue-specific would have been detected, if they were

present on the immunizing cell line. We conclude that more restricted antigens, possibly: 1) Do not exist; 2) Are not readily detected by current methods; or, 3) Are not present on cell lines. The few ovarian carcinoma cell lines are not likely to be representative of in vivo tumors; the difficulty in establishing new lines of ovarian and other carcinomas (except renal) is well known. In this paper we present our results of 2 initial fusions using fresh tissue as immunogen and screening target, and we believe further similar studies will be productive. The strategy of freezing uncloned hybridomas, to allow time for specificity testing on frozen sections, made this approach possible, and should be widely applicable.

Example II

Example for Therapy

We have attempted to select monoclonal antibodies that might also be effective agents for diagnosis and intraperitoneal therapy or radioimmunodetection of human ovarian carcinoma. Antibodies were tested for reactivity with the surface of fresh ovarian carcinoma ascites cells, and for non-reactivity with normal mesothelial cells. The antibodies tested included 33 that had been identified previously as reacting with epithelial differentiation antigens. Five antibodies were selected with the desired

specificity, MH99, MT179, MW162, MW207 and MX35, and these antibodies also reacted with cryostat sections of most of all ovarian carcinomas and benign ovarian cysts. All reacted also with certain normal epithelial cells. We also observed that the degree of heterogeneity of antigen expression on ascites carcinoma cells was dependent on the particular antigen being examined, and related to the biochemical nature of the antigen. In particular, most ABO blood group-related antigens showed a striking degree of heterogeneity. The rationale for intraperitoneal immunotherapy and the criteria for selecting appropriate antibodies are discussed.

The effectiveness of monoclonal antibodies in cancer immunotherapy and immunolocalization depends on their specificity. The optimal target antigen would be tumor-specific, and much effort has been directed to obtain such monoclonal antibodies. To date, no monoclonal antibody has been conclusively shown to identify a tumor-specific antigen; though there are some possible candidates for breast carcinoma (Schlom, J., et al. (1985) Adv. Cancer Res., 43:143-174), ovarian carcinoma (Tsuji, Y., et al. (1985) Cancer Res., 45:2358-2362), pancreatic carcinoma (Chin, J., et al. (1985) Cancer Res., 45:1723-1729) and lung

small cell carcinoma (Tong, A.W., et al. (1984) Cancer Res., 44:4987-4992), but characterization of these antigens is still preliminary. The search for tumor-specific antigens is based on numerous prior immunological studies suggesting the presence of such antigens on human tumors (Old, L.J., et al. (1981) Cancer Res., 41:361-375; Herberman, R.B., et al. (1974) Adv. Cancer Res., 19:207-263; Kedar, E., et al. (1983) Adv. Cancer Res., 38:171-288; Szigeti, R., et al. (1985) Adv. Cancer Res., 43:241-306; Shuster, J.,e t al. (1980) Prog. Exp. Tumor Res., 25:89-139; and Thomson, D.M.P., et al. (1985) Int. J. Cancer 35:707-14). The published evidence primarily consists of data indicating an immune response to syngeneic tumors, as detected by assays for antibodies (Old, L.J., et al. (1981) Supra), lymphocyte-mediated growth inhibition or cytotoxicity (Herberman, R.B., et al. (1974) Supra), T lyymphocyte-mediated cytotosicity (Kedar, E., et al. (1983) Supra), delayed hypersensitivity (Herberman, R.B., et al. (1974) Supra), macrophage migration inhibition (Szigeti, R., et al. (1985) Supra) or leukocyte adherence inhibition (Shuster, J., et al. (1980) Supra and Thomson, D.M.P., et al. (1985) Supra). However, such approaches have not yet allowed definite characterization of any tumor antigen, and the results have generally not been consistently reproduced in different laboratories, so the presence of human

tumor-specific antigens must still be considered speculative (discussed in references Schlom, J., et al. (1985) Supra; Weiss, D.W., et al. (1980) Curr. Topics Micro. Immunol. 89:1-83; North, R.J., et al. (1984) Adv. Immunol., 35:89-156).

Although tumor-specific monoclonal antibodies are not available, monoclonal antibodies to differentiation antigens may be of value. Such Abs react with certain normal adult cells as well as tumor cells of particular types, so toxicity arising from reactivity with normal cells is probable with some of all of such monoclonal antibodies. However, by adjusting the dose and by modifying the monoclonal antibody (such as by preparation of antibody fragments, or conjugates with radioisotopes or toxins) it may be possible to obtain a therapeutic effect without major side effects. It should be considered that such monoclonal antibodies are more specific than current therapeutic agents. Chemotherapeutic agents were selected initially not for their specificity, but for their toxicity, and effective treatment requires the maximum tolerated dose; the same approach seems valid for mAB therapy. The major difference in this regard between monoclonal antibodies and new chemotherapeutic drugs is that monoclonal antibodies cannot be pretested in animals; this makes determination of the optimal treatment regimen much more difficult.

We have focused on selecting mABs for therapy and radioimmunodiagnosis of ovarian carcinoma. This tumor type is the leading cause of death among patients in the United States with gynecologic malignancy, and is not treated effectively by current methods (Bender, H.G., and Beck, L. (Eds) (1983) Carcinoma of the Ovary. New York:Gustav Fischer Verlag, 1983 and Haije, W.G., et al. (1982) Ann. Clin. Biochem., 19:258-262). Radioimmunodetection might decrease the need for "second-look" surgery, which is currently performed routinely to diagnose tumor recurrence. In regard to monoclonal antibody therapy, ovarian carcinoma has two major advantages. First, as with other non-essential organs, a tissue-specific antigen would be as useful as a tumor-specific antigen. This advantage however has not yet materialized, in that no differentiation antigen specific for ovarian epithelial cells has yet been described. Second, the tumor grow primarily in the peritoneal cavity; blood-borne metastases can occur, but most patients succumb prior to this (Bergman, F., et al. (1966) Acta. Obstet. Gynecol. Scand., 45:211-225). Therefore, intraperitoneal therapy wold be expected to enhance interaction of the monoclonal antibody with tumor cells and to reduce interaction with normal, antigen-positive cells outside the peritoneal cavity. The importance of this factor is difficult to evaluate at

present. Serum proteins pass rapidly from the peritoneal cavity to the blood in normal animals (French, J.E., et al. (1960) Quart. J. Exper. Physiol., 45:88-103).

Antibodies injected i.p. into patients with ovarian carcinoma are initially exposed to only one type of normal cell, mesothelial cells, which line all surfaces of the peritoneal cavity. In this paper we describe the selection of monoclonal antibodies that react with the surface of fresh tumor cells but not with normal mesothelium. Fresh ascites carinoma cells were used as targets in immunofluorescence. Ovarian carcinomas grow as both ascites and solid modules of tumor cells attached to the lining of the peritoneal cavity. To predict reactivity of monoclonal antibodies with tumor cells in vivo, use of ascites cells as targets seems more reliable than examining either tissue culture lines of ovarian carcinoma (which are rare and probably not representative) or frozen sections of fresh tumors (which would not indicate which antigens are accessible on the cell surface in vivo). Mesothelial cells were examined in frozen sections. Also examined were frozen sections of solid ovarian carcinomas and benign ovarian cysts. The monoclonal antibodies tested were 33 Abs produced by our laboratories which, from previous studies, appeared to demonstrate specificity for epithelial cells,

and included monoclonal antibodies reacting with ABO blood group-related antigens. We have identified 5 distinct monoclonal antibodies that reacted with the surface of most or all fresh tumor specimens and that were negative on mesothelium; they also reacted with various normal epithelial cells. These antibodies appear suitable for further evaluation as potential therapeutic agents.

Antibodies. The 33 monoclonal antibodies tested in this study were generated and characterized previously. They were obtained from mice immunized with various human and were included in these experiments on the basis of preferential reactivity with epithelial cells in frozen sections of normal human tissues and in cultured cell lines. All have been tested on a wide range of cell lines and normal tissues, and also have been partially characterized biochemically by immunoprecipitation (which in some cases did not precipitate a detectable component). Based on these data, the monoclonal antibodies appeared different from each other. In the following list, they are grouped according to the type of carcinoma used for immunization and to the publication in which they are described: ovarian, MF61, MF116, MH55, MH94 (Mattes, M.J., et al. (1984) Proc. Natl. Acad. Sci. USA 81:568-572; Cordon-Cardo, C., et al. (1985) Int. J. Gynecol. Pathol., 4:121-130), MH99 (Mattes, M.J., et

al. (1983) Hybridoma 2:253-264), MQ49, MT334 (Mattes, M.J., et al. (1985) J. Histochem. Cytochem., 33:1095-1102), MT179, MW162, MW207, MX35 (Mattes, M.J., et al. Four mouse monoclonal antibodies to human epithelial differentiation antigens above), MR54, MT78, MV9 (M.J. Mattes, unpublished data); bladder, T16, T87 (Fradet, Y., et al. (1984) Proc. Nat. Acad. Sci. (Wash.), 81:224-228); renal, S6 (Ueda, R., et al. (1981) Proc. Nat. Acad. Sci. (USA), 78:5122-5126); teratocarcinoma, K4 (Rettig, W.J., et al. (1985) in press); choriocarcinoma, LK26, SV19, SV63 (placental alkaline phosphatase) (Rettig, W., et al. (1985) Int. J. Cancer 35:469-475); lung, F-15, F-16 (J. Feikert, unpublished data and U.S. patent application S.N. 474,225); and colon, HT29-15, V-215, CLK314 (Sakamoto, J., et al. (1985) Fed. Proc. 42:792). We also tested 7 monoclonal antibodies to ABO blood group-related antigens, since these are epithelial differentiation antigens. These monoclonal antibodies also were obtained after immunizing mice with various human carcinomas, and include: anti-A, HT29-36 (Furukawa, K., et al. J. Immunol., in press); anti-B (Ueda, R., et al. (1981) Supra) anti-Lewisa, T174; anti-Lewisb, T218; anti-H type 2, H11; anti-X, P12; and anti-Y, F3 (Sakamoto, J., et al. (1984) Molecular Immunol., 21:1093-1098).

Ascites cells. 0.5-2.0 l ascites fluid from patients with serous adenocarcinoma of the ovary were

filtered through 4-ply gauze and spun 5 min at 600g. Pelleted cells were resuspended in 5-10 volumes of supernatant, and 40 ml aliquots were underlaid with 10 ml Ficoll-Paque (Pharmacia, Piscataway, NJ). After spinning 15 min at 3,000g, the cells at the interface were collected, washed once with Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, N.Y.), 7.5% fetal calf serum, 10% dimethylsulfoxide at various concentrations (1-10% packed cell volume/volume) and frozen in liquid nitrogen. Immunofluorescent staining was performed by standard procedures (Mattes, M.J., et al. (1984) Proc. Natl. Acad. Sci. USA 81:568-572), using mAb sera at 1/50, fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) at 1/40 in medium containing 20% normal human serum, and 3-6 ul packed ascites cells per sample. Examination was by epi-illumination using a 75W xenon lamp, Leitz filter cube H, and a 40x objective. Some samples contained normal cells such as lymphocytes, macrophages and mesothelial cells as well as tumor cells; therefore, evaluation was based on observation of clustered cells only, since carcinoma cells in ascites usually are in clusters, while the normal cells present are rarely in clusters. Samples of cells were processed and examined by the Pathology Laboratory at SKI (Sloan-Kettering Institute, N.Y., N.Y.) (Dr. Patricia Saigo), to confirm that virtually

all cells in clusters were malignant. Photographs of immunofluorescence were prepared using Kodak Tri-X film and 90 second exposures. Bone marrow cells from normal donors were provided by the Bone Marrow Transplantation unit at SKI and examined similarly by immunofluorescence.

Cryostat sections. General methods for staining 0.007 mm sections by the avidin-biotin complex method have been described (Mattes, M.J., et al. (1985) J. Histochem. Cytochem. 33:1095-1102). Ovarian cysts included 1 serous cystadenoma, 3 mucinous cystadenomas, 1 simple cyst, 1 serous cystadenocarcinoma of low malignant potential, and 1 mucinous cystadenocarcinoma of low malignant potential. Strips of the cyst wall were folded in pleats before freezing. Tissues containing normal mesothelium included the diaphragm, body wall and pericardium. Ovarian carcinomas included serous (7 specimens), mucinous (1 specimen) and endometroid (2 specimens).

The 33 monoclonal antibodies evaluated were generated in our laboratories by immunization with ovarian, bladder, renal, lung, and colon carcinomas and with choriocarcinomas. We anticipated that the expression of cell surface antigens on ovarian carcinoma ascites cells might be quite different from antigen expression on cultured

cell lines, and therefore we tested 26 monoclonal antibodies reacting with a variety of epithelial differentiation antigens. ABO blood group-related antigens are epithelial differentiation antigens; therefore, a panel of 7 monoclonal antibodies to blood group-related structures, which were also obtained after immunizing mice with human carcinomas, was included.

As the initial screening for a most of the monoclonal antibodies, we examined frozen sections of a benign ovarian cyst, to confirm the specificity of the monoclonal antibodies for epithelial cells. A benign cyst was used rather than a normal ovary because, in our experience, epithelial cells are better preserved in frozen sections. Monoclonal antibodies reacting with connective tissue or blood vessels were eliminated at this stage, which included 8 monoclonal antibodies (MR54, T16, T87, V-215, CLK314, F-15, F-16 and LK29). None monoclonal antibodies produced the expected staining of epithelial cells only. Eight monoclonal antibodies were negative on the benign cyst, but were tested further for reactivity with fresh ascites cells. We observed that some monoclonal antibodies stained the outer surface of the cyst as well as the inner epithelial cells. The cells lining the outer surface are presumably drived from the normal ovarian epithelium, but

due to cyst formation they appear flattened, like mesothelial cells, in morphology. The monoclonal antibodies that stained the outer as well as the inner surface of the cyst, (MH94, MQ49, MT334, HT29-15 and F3), all react with heat stable (100°) antigens, which are probably carbohydrate. One, F3, reacts with the Y blood group-related antigen. Another, MQ49, reacts with both glycolipids and mucin-like molecules (Mattes, M.J., et al. (1985) Supra). MH94, MT334 and HT29-15 also react with mucin-like molecules. The MH94 antigen was detected by immunoprecipitation after labeling with $H_2{}^{35}SO_4$, but not with [3H]glucosamine (Mattes, M.J., et al. Proc. Nat'l. Acad. Sci USA 81:568 (1984) and unpublished data). Other monoclonal antibodies (MH99, MT179, K4 and SV19) reacted with cells lining the inner surface of the cyst only.

The 25 remaining monoclonal antibodies were tested against at least 2 specimens of fresh ovarian carcinoma ascites cells, by immunofluorescence. We had initially attempted, to facilitate assays, to attach these target cells to wells of Terasaki plates using concanavalin A, which has been effective with a wide range of nonadherent cell types (Mattes, M.J. et al., J. Immunol. Meth. 61:145 (1983). However, the ascites cells did not attach stably under these conditions, suggesting that they have unusual

surface properties. We also tested 2 broadly reactive monoclonal antibodies, MA103 and AJ2 (Mattes, M.J., et al. (1983) Hybridoma 2:253), which are present on all human tissue culture cells: these monoclonal antibodies reacted with all ascites cells tested. Monoclonal antibodies negative with the 2 ascites specimens were not tested further (11 monoclonal antibodies: MF61, MF116, MH55, MV9, K4, SV19, SV63, S6, HT29-15, HT29-36 and S8), but positive monoclonal antibodies were tested on additional ascites specimens. Results are summarized in Table 3. Six monoclonal antibodies reacted with 5/5 or 4/5 specimens, and produced fairly homogeneous, ringed staining of tumor cells 2E). In these studies, we examined primarily clumped tumor cells since these were identified as malignant. The nature of the single cells was more variable, but many specimens clearly contained many malignant single cells, as indicated both by morphology and immunofluorescent staining.

Several monoclonal antibodies produced staining with certain unusual characteristics: 1) Tumor cells had a striking degree of heterogeneity. The fraction of positive tumor cells (in clumps) ranged from a few per cent to 50%. Positive cells were often extremely bright, although brightness was variable, so the positive and negative cell populations appeared to be distinct. A single clump of

cells usually contained a mixture of strongly positive and negative cells. 2) In some specimens, many positive cells were not ringed, but were stained over a continuous portion, generally 1/3 to 2/3, of their surface. This occurred in both clumped cells and single tumor cells. In clumped cells, the stained portion of the membrane was usually the area not in contact with another cell. This antigen distribution did not appear to be antibody-mediated, since it occurred in cells stained at 4° in the presence of 10 mM NaN3 (and which had been pretreated for 30 min with NaN3 before the first antibody incubation).

Of the monoclonal antibodies to ABO blood group-related antigens tested, most that were positive on asciates cells produced this heterogeneous pattern, and this occurred with most specimens examined. The only other monoclonal antibodies producing this heterogeneous pattern were MQ49 and HT29-15, which also recognize carbohydrate determinants (Mattes, M.J., et al. (1985) J. Histochem. Cytochem. 33:1095 Supra and Sakamoto, J., et al. (1985) Fed. Proc., 42:792). The exception among blood group-related monoclonal antibodies was F3 (anti-Y) which produced bright, ringed staining of most or all tumor cells in all specimens examined. This antigen, however, is present on erythrocytes (strongly on type O, weakly on types A & B) (Sakamoto, J.,

et al. (1984) Molecular Immunol., 21:1093-1098). Therefore, it does not appear to be a suitable target for tumor localization or therapy and has not been included in our subsequent studies of monoclonal antibody specificity.

The 5 monoclonal antibodies consistently reactive with ascites cells were tested further for specificity and results are included in Table 3. They reacted with frozen sections of most of all ovarian carcinomas tested, and with the epithelial cells of many benign ovarian cysts. The most consistently positive monoclonal antibody was MH99, which reacted without exception with all cells derived form the ovarian epithelium. Table 4 summarizes the normal tissue reactivity of these monoclonals, which was described above. MH99 is most consistently positive and reacts with nearly all normal epithelial cells. THe other mAbs react with a subset of normal epithelial cells. Normal mesothelial cells in frozen sections of the lower surface of the diaphragm, body wall and pericardium were negative with all 5 mAbs. As described (Mattes, M.J., et al. (1983) Hybridoma 2:253 and "four mouse monoclonal antibodies to human epithelial differentiation antigens" Proc. Natl. Acad. Sci. USA) blood leukocytes and erythrocytes were negative by immunofluorescence. Normal bone marrow cells, examined by immunofluorescence, were also negative.

The pattern of staining in frozen sections varied depending on the particular antibody. Antibodies MH99, MT179, and Mw207 stained all sides of the cells equally, in a pattern suggestive of membrane staining. In contrast, antibodies MW162 and MX35 often stained only the luminal edge of the cells and sometimes also produced a granular staining pattern.

To determine whether antibody-induced capping or other type of modulation of surface antigens occurs, immunofluorescent staining of 2 ascites specimens was performed in the absence of NaN3. Following staining, cells were incubated for 45 min at 37. This treatment had no effect on antigen distribution, relative to a control stained in the presence of NaN3, with any of the 5 monoclonal antibodies tested. We conclude that capping does not readily occur.

We also investigated the possibility that large amounts of soluble antigen in ascites fluid might inhibit monoclonal antibody binding in vivo. Fifty ul of autologous ascites fluid, collected at the same time as the cell specimen, was included during the first antibody incubation. This produced no detectable inhibition of immunofluorescent staining with any of the 5 monoclonal antibodies tested,

suggesting that such inhibition would not occur in vivo.

We have identified 5 monoclonal antibodies that have potential value in intraperitoneal immunotherapy or immunodiagnosis of ovarian carcinoma, since they react with the surface of fresh tumor cells but are negative on normal mesothelial cells. We attempted to examine cells as similar as possible to the cells that would be encountered by a monoclonal antibody injected i.p. We plan to examine cells from biopsies obtained following injection of radio-labeled antibody, to confirm the reactivity of the monoclonal antibodies in vivo. It is evident that examination of ovarian carcinoma cell lines, or cryostat sections of fresh tumors, does not provide reliable information regarding antigen expression on the surface of cells in vivo. For example, MT334 reacted with frozen sections of 10/10 tumors, but reacted weakly with only 1/5 ascites specimens.

The monoclonal antibodies described all react with some normal adult cells, but they may be effective in tumor localization or therapy without producing unacceptable side-effects. It must be considered that all current forms of cancer therapy are toxic, and the antibodies described are more specific than effects of chemotherapy or radiation. Moreover, after i.p. injection it is uncertain how

efficiently the antibodies will reach the positive normal cells. For example, antigens located at the luminal edge of epithelial cells, such as MW162 and MX35, may not be exposed to the circulating antibodies. Also IgM's such as MW162 may not penetrate tissues sufficiently to reach the positive normal cells. If the antibody is fragmented or conjugated to other molecules, this may strongly affect the localization in normal tissues and the side effects. In addition, monoclonal antibody binding to normal tissues may or may not produce toxicity. Radioisotope-conjugated monoclonal antibodies may damage tumor cells, which are relatively radio-sensitive, much more readily than normal cells to which they bind. Experimentation in humans is required to resolve these questions. Initially the localization of radiolabeled antibodies will be investigated to determine whether the monoclonal antibodies bind effectively to tumor and/or normal tissues. These experiments will also indicate whether the monoclonal antibodies might be useful for radioimmunodetection of small tumor masses, which might eliminate in some cases the need for "second-look" surgery to detect tumor recurrence.

The answer to this question is obcured by a large number of reports describing putative tumor-specific antigens (Old, L.J., (1981) Supra; Herberman, R.B. (1974) Supra; Kedar, E., et al. (1983) Supra; Szigeti, R. (1985) Supra; Shuster, J., et al. (1980) Supra; Thomson, D.M.P., et al. (1985) Supra; Weiss, D.W., (1980) Supra and North, R.J. (1984) Supra). Until such antigens have been well-characterized, which has not been achieved in a single case, they must be considered as speculative. A more tissue-specific monoclonal antibody, reactive with fewer normal cells, is clearly preferable, but it should be considered that ovarian epithelial cells are relatively undifferentiated, and produce no known specific differentiation marker. Moreover, the truly tissue-specific markers, such as proteins secreted by the prostate, pancreas or breast, are often lost as a tumor progresses to a less differentiated state. We emphasize that the choice of the optimal target antigen for therapy depends not only on its restricted distribution. Equally important is its consistent expression on nearly all tumor cells, at least from a particular patient, as well as on a low frequency of antigen-negative variants.

The monoclonal antibodies used in these studies were generated in our laboratories, and may or may not be the optimal monoclonal antibodies for i.p. immunotherapy.

Other laboratories also have attempted to generate monoclonal antibodies specific for ovarian carcinoma. Bast et. al. (Kabawat, S.E., et al. (1983) Int. J. Gynecol. Pathol., 2:275-285) described monoclonal antibody OC125, which recognizes a high molecular weight antigen that appears to be a valuable serum marker for ovarian carcinoma (Klung, T.L., et al. (1984) Cancer Res., 44:1048-1053). In cryostat sections of normal tissues, only the epithelia of the uterus, fallopian tube and endocervix, and mesothelial cells of the peritoneum, pleura and pericardium were positive. Approximately 85% of ovarian serous carcinomas were positive. The reactivity with normal mesothelial cells causes this monoclonal antibody to be apparently, inappropriate for i.p. immunotherapy. Also, expression of OC125 appeared to be heterogeneous in cryostat sections, with considerable negative cells being detected in positive tumors (Kabawat, S.E., et al. (1983) Amer. J. Clin. Pathol. 79:98-104). Gangopadhyay et al. (Gangopadhyay, A., et al. (1985) Cancer Res., 45:1744-1752) described the 1D3 monoclonal antibody, which reacted with essentially all ovarian mucinous carcinomas, and with only the normal colon among normal tissues examined; however, the more common serous carcinomas were negative. Tagliabue et al. (Tagliabue, E., et al. (1985) Cancer Res., 45:379-385) obtained 2 monoclonal antibodies to ovarian carcinoma, MOv1

and MOv2. Mov2 reacted with most but not all ovarian carcinomas of all types, and with normal colon, stomach and breast. It reacted by immunofluorescence with most but not all fresh ovarian carcinoma ascites cells. MOv1 reacted with mucinous but not serous ovarian carcinomas, and also reacted with the normal colon. Tsuji et al. (Tsuji, Y., et al. (1985) Supra) recently described 2 monoclonal antibodies: 4C7 reacted with most ovarian mucinous, endometroid and mesonephroid, but not serous carcinomas, while 3C2 reacted with most serous and endometroid, but not mucinous or mesonephroid carcinomas. These 2 antibodies did not react with any normal tissues, benign ovarian tumors or carcinomas of other organs.

Three monoclonal antibodies obtained to breast-carcinoma-associated antigens have been found to react with ovarian carcinoma ascites cells. F36/22 reacted with 47/47 ascites specimens, and was negative with normal mesothelial cells in the same specimens (Croghan, G.A., et al. (1984) Cancer Res., 44:1954-1962). This monoclonal antibody also reacted with sections of ovarian carcinomas, but not with the normal ovarian epithelium, while benign ovarian tumors had weak and variable staining. However, many normal cells were positive with F36/22, including the breast, lung, sebaceous gland, sweat gland, uterus and

kidney. Monoclonal antibody 3.14.A3, later called HMFG2, reacted with ovarian carcinomas and with normal bile duct, pancreas, sebaceous bland, salivary gland, kidney, lung, sweat gland and uterus (Arklie, J., et al. (1981) Int. J. Cancer, 28:23-29). This antibody, 131-I-labeled, was used in preliminary studies of i.p. injection for therapy of ovarian carcinomas (Epenetos, A.A., (1984) The Lancet, 1441-1443).

B72.3 has also been detected in fresh ovarian carcinoma ascites cells (Johnston, W.U.V., et al. Cancer Res. 45:1894 (1985). It is positive on breast and other carcinomas, but negative on all normal tissues examined. In sections of breast carcinomas, there was marked heterogeneity in the expression of B72.3 (Schlom, J. et al. (1985) Supra). The presence of other well-characterized tumor markers, including carcinoembryonic antigen, alpha-fetoprotein and human chorionic gonadotropin, was also investigated in ovarian carcinomas; all were negative on more than 75% of serous carcinoma (Casper, S., et al. Am. J. Obstet. Gynecol. 149:154 (1984).

An interesting observation was that many monoclonal antibodies to carbohydrate antigens produced striking heterogeneity in their staining of ascites cells. Tumor heterogeneity is of course a major obstacle in effective treatment, and our observations suggest the important point that the level of heterogeneity can vary widely depending on the particular antigen, and that this level may be related consistently with the biochemical nature of the antigen. The mechanism for variation in blood group antigen expression is unknown, but may be related to the altered and variable state of differentiation of tumor cells. We suggest that this heterogeneity is directly related to the fact that blood group carbohydrate antigens, including ABO, Ii and T, account for many of the most consistent differences between malignant and normal cells of the same histological type (Feizi, T., (1985) Nature 314:53-57). That is, expression of carbohydrate antigens may be a very sensitive indicator of the state of differentiation of a cell, allowing such antigens to be useful in diagnosis of malignancy. But, for the same reason, malignant cells may generally vary in expression of these antigens, making them unsuitable as targets for immunotherapy.

The rationale for i.p. immunotherapy is complex. Intraperitoneal therapy of ovarian carcinoma has been used extensively with a colloidal suspension of Cr32P04 (Rosenshein, N.B., et al. (1979) Obstet. Gyneco. Survey, 34:708-720) or with other chemotherapeutic drugs (Markman, M. in S.B. Howell (ed.) Intra-Arterial and Intracavitary Cancer Chemotherapy, PP. 61-69 Boston: Martiners Nijhoff, 1984), though evidently often without an understanding of the physiology of efflux from the peritoneal cavity, as noted by Leichner et al. (Leichner, P.K., et al. (1980) Radiology 134:729-734). Efflux of substances from the peritoneal cavity, including proteins, colloids, and erythrocytes, is very rapid via lymphatics of the lower surface of the diaphragm. The lack of a substantial barrier to efflux is due to the presence of specilized mesothelial cells and lymphatics at this location (French, J.E., et al. (1960) Quart. J. Exper. Physiol., 45:88-103). This factor reduces the advantage of intraperitoneal therapy. However, there are several factors that can enhance the effectiveness of this approach. First, as noted for drug therapy (Markman, M., (1984) In: S.B. Howell (ed.), Intra-Arterial and Intracavitary Cancer Chemotherapy, pp. 61-69. Boston: Martinus Nijhoff), the relative concentration in peritoneal fluid and in blood depends on both the rate of efflux from the peritoneal cavity and the rate of clearance from the

blood. Substances cleared rapidly from the blood are therefore preferable. The clearance rate of monoclonal antibodies from the blood might be increased in various ways, such as removing sialic acid (Ashwell, G., et al. (1974) Adv. Enzymol., 41:99-128). Second, patients with ovarian cancer have impaired efflux from the peritoneal cavity, due probably to blockage of lymphatics by tumor cells, which is presumably the reason for development of ascites (Feldman, G.B., et al. (1972) Cancer Res., 32:1663-1666 and Coates, G., et al. (1973) Radiology, 107:577-583). Third, it might be possible to decrease the rate of efflux in various ways which have been effective in experimental animals, such as by anaesthesia, blocking major lymphatic vessels (Courtice, F.C., et al. (1951) Austral. J. Exper. Biol. Med. Sci. 29:451-458), or disrupting the lymphatic capillaries on the lower diaphragm (Raybuck, H.E., et al. (1950) Am. J. Physiol. 199:1021-1024). Fourth, since the toxicity of monoclonal antibodies will result from binding to normal epithelial cells, if the antibody can be confined to the vascular system after it enters the blood, toxicity may be eliminated. This might be achieved by conjugating IgG to particles of diameter greater than 0.1u, which are too large to pass through the capillary endothelium (Renkin, E.M., et al. (1977) Circ. Res., 41:735-743 and Simionescu, N., et al. (1972) J. Cell Biol.

53:365-392). Regardless of the method of administration, some of the monoclonal antibody will enter the blood; this may be useful in that potentially metastatic tumor cells in the blood will be eliminated. The method developed for i.p. chemotherapy is to inject a large volume, and to withdraw it after several hours (Markman, M., (1984) Supra); this seems equally appropriate for monoclonal antibody therapy. Intraperitoneal immunotherapy for ovarian cancer has previously been attempted with polyclonal (Order, S.E., et. al. (1981) Cancer 48:590-596) and monoclonal (Epenetos, A.A., (1984) The Lancet, 1441-1443) antibodies in preliminary experiments. Clearly many variables must be investigated in order to devise the optimal approach.

These hybridoma cell lines are on deposit at Sloan-Kettering Institute 1275 York Avenue, New York, NY and at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, a recognized international depository, under the following designations:

SKI Number	ATCC Designation	ATCC Deposit Date
MF116	HB 8411	October 28, 1983
MD144	HB 8409	October 28, 1983
MH55	HB 8412	October 28, 1983

SKI Number	ATCC Designation	ATCC Deposit Date
MF61	HB 8410	October 28, 1983
MH94	HB 8413	October 28, 1983
MH99	HB 8406	October 28, 1983
ME195	HB 8431	November 16, 1983
ME46	HB8430	November 16, 1983
MW162		
MT179		
MW207		
MT35		
MU78	HB8877	July 15, 1985
MT334	HB8876	July 15, 1985
MQ49	HB8874	July 15, 1985

Table 1

Reactivity of moAbs with cryostat section of normal human tissues[a]

Tissue	MT179	MW162	MW207	MX35
Esophagus	-	+	-	-
Stomach	-	+	-	-
Colon	+	-	-	-
Epiglottis	nd[b]	-	-	-
Bronchus	nd	+	+	+
Lung	+	+	+	+
Skin	+	-	-	-
Kidney	-	+[c]	+[c]	+[c]
Pancreas	+	+	+	-
Liver	-	-	-	-
Thyroid	-	+	+	+
Ovary	-	-	-	-
Uterus	-	+	+	+
Breast	+	+	+	-
Testes	-	-	-	-
Heart	nd	-	-	-
Spleen	-	-	-	-
Lymph node	-	-	-	-
Thymus	-	-	-	-
Brain	-	nd	nd	nd

[a]The positive cells with these Abs were exclusively epithelial cells.

[b]Not determined

[c]In the kidney, MW207 reacted with proximal tubules, MW162 with Henle's loop and distal tubules, and MX35 with collecting tubules.

Table 2

Reactivity[a] of moAbs with tissue culture cell lines

Cell Type	MT179	MW162	MW207	MX35
Carcinomas				
Ovarian	3/8[b]	5/8	5/8	3/8
Uterine	2/2	0/2	0/2	0/2
Colon	9/11	2/9	5/9	0/9
Bladder	5/9	3/6	1/6	0/6
Pancreas	3/4	2/3	2/3	0/3
Lung	5/11	6/8	6/8	1/8
Breast	2/5	4/4	3/4	0/4
Renal	2/17	2/11	10/11	3/11
Prostate	1/2	0/1	0/1	0/1
Bile duct	1/1	-	-	-
Melanomas	0/21	0/10	0/10	0/10
Astrocytomas	1/17	0/10	2/10	0/10
Sarcomas	-	0/4	1/4	0/4
Teratocarcinomas	-	0/1	1/1	0/1
Choriocarcinomas	1/1	1/1	1/1	0/1
Neuroblastomas	0/5	-	-	-
Hematopoietic tumors				
T lymphocyte	0/8	0/7	0/7	0/7
B lymphocyte	0/9	0/4	0/3	0/4
Null lymphocyte	0/6	0/4	0/4	0/4
Myeloid	0/3	0/2	0/2	0/2
Monocytoid	-	0/2	0/1	0/2
Myeloma	-	0/1	0/1	0/1
Normal cells				
Fibroblasts	0/3	0/4	0/4	0/4
Kidneyepithelial	2/3	3/3	2/3	1/3

[a]MW207 was tested in a rosetting assay for cell surface antigens using a starting dilution of 1/1000. MT179, MW162 and MX35 were tested in a peroxidase assay on fixed, permeabilized cells, using a starting dilution of 1/500.

[b]Number positive/number different cell lines tested. The individual positive cell lines and the titers are listed in the text.

Table 3

Reactivity of moAbs with ovarian epithelial tumors

		Ascites Cells		Frozen Sections	
MoAb	Determinant	Positive	Heterogeneous	Benign Cyst Epithelia[c]	Carcinomas[d]
MH99	P[a]	5/5[b]	no	7/7	10/10
MT73	P	2/5	yes,10-50%	---	-----
MT179	?	4/5	no	5/5	8/10
MW207	?	5/5	no	3/6	10/10
MX35	?	5/5	no	3/6	10/10
MH94	C	1/5	yes,20%	4/6	4/9
MQ49	C	3/5	yes,10-50%	1/1	6/8
MT334	C	1/5	yes,30%	4/5	10/10
MW162	C	5/5	no	3/6	10/10
HT29-15	C	1/2	yes,5%	1/1	-----
H-11	C,H type 2	1/2	yes,2%	---	-----
T164	C,Le^a	1/2	yes,2%	---	-----
T218	C,Le^b	1/2	yes,1%	---	-----
F3	C,Y	5/5	no	1/1	-----
P12	C,X	3/5	yes,2-50%	0/1	-----

[a] P, protein; C, carbohydrate; ?, unknown.

[b] Number positive/number specimens examined.

[c] % tumor cells positive.

[d] Benign cysts included 1 serous cystadenoma, 3 mucinous cystadenomas, 1 simple cyst, 1 serous cystadenocarcinoma of low malignant potential, and 1 mucinous cytadenocarcinoma of low malignant potential. Ovarian carcinomas included serous (7 specimens), mucinous (1) and endometroid (2).

Table 4

Reactivity of MoAbs with normal epithelial cells

MoAb	Positive Tissues
MW99	most or all epithelial cells
MC179	colon, lung, skin, pancreas, breast
MW162	esophagus, stomach, bronchus, uterus, lung, kidney[a], pancreas, thyroid, breast
MW207	bronchus, lung, kidney[a], pancreas, thyroid, uterus, breast
MX35	bronchus, lung, kidney[a], uterus, thyroid

[a]In the kidney, MW207 reacted with proximal tubules, MW162 with Henle's loop and distal tubules, and MX35 with collecting tubules.

Radiolabeled monoclonal antibodies (Mabs) or their fragments are receiving considerable attention as tumor targeting agents for a wide variety of solid tumors (Epenetos et al., 1982; Larson et al., 1983; Mach et al., 1983; Baum et al., 1986; Delaloye et al., 1986; Chatal et al., 1987; Sharkey et al., 1990; Lloyd et al., 1993). Although labeled Mabs are beginning to have an impact in the clinical domain, their specificity and sensitivity for tumor detection still remain to be improved. Radioimmunotargeting with $F(ab')_2$ and Fab fragments of antibodies has generally been favored over the use of the intact Ig due to their shorter biological half-life in the blood and rapid tissue distribution and clearance of the fragments resulting in higher tumor to normal tissue and tumor to blood ratios (Fjeld et al., 1992).

The primary objective of our work was to study the biodistribution and pharmacokinetics of radiolabeled Mab MX-35 in a xenograft model of human ovarian cancer as a preliminary step to clinical studies using this antibody. Mab MX-35 (IgG1) was developed by immunization of mice with ascites and solid tumor ovarian cancer cells (Mattes et al., 1987; Mattes et al., 1989). It reacts strongly and relatively uniformly with 75-80% of ovarian carcinoma samples and with a few normal tissues (Rubin et al., 1989, Rubin et al., 1991). The antigen detected is a cell surface 90,000 dalton, non-secreted glycoprotein (M. Welshinger, B.Y.T. Yin and K.O. Lloyd, unpublished data).

In this study we place special emphasis on radiolabeled $F(ab')_2$ fragments of Mab MX35 as an alternative to the intact antibody. By comparing the tissue distribution of radiolabeled intact IgG with $F(ab')_2$ fragments we demonstrated much better targeting using the fragments. More interestingly, compared with intact antibody, the $F(ab')_2$ fragments gave markedly increased values of absolute tumor uptake of the antibody. This result contrasts with previous results (Sands, 1990; Gerretsen et al., 1991; Molthoff et al., 1992) obtained from comparative experiments.

Materials and Methods

Monoclonal Antibody Production

Monoclonal antibody MX-35, a murine IgG1, was produced from hybridoma ascites grown in BALB/c mice. High titer ascites batches were pooled for purification. MAb MX-35 was purified from the ascites through several steps including removal of lipoprotein by ultracentrifugation at 100,000 g and ammonium sulfate precipitation (50% saturation). Final purification was by protein A-agarose chromatography (Ey et al., 1978).

Preparation of Fragments

Following overnight dialysis of purified MX-35 immunoglobulin in 25mM sodium acetate pH 4.5, 25 μl of pepsin (1.5 mg ml^{-1}; Sigma Chemical Co., St. Louis, MO) was added to the antibody (2 mg in 400 μl) and incubated overnight at 37 C. $F(ab')_2$ fragments were isolated using a kit obtained from Bio Chrom International, Tustin, CA. High yield binding buffer (250 μl) containing 30 μl of anti-pepsin was added to the antibody-pepsin combination. The entire sample was diluted with 220 μl of high yield binding buffer and centrifuged to 2000 g for 15 min and the supernatant was placed on a protein-A-Avidchrome column. The unadsorbed fraction was concentrated using a Centricon 30 unit (Amicon, Beverly, MA) at 1075 g at 4 C. The final antibody concentration obtained was 1.95-2.34 mg ml^{-1} and the overall yield ranged from 63-65%. The identity of the fragments was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Staining with Coomassie blue, revealed bands of 23 kD and 25 kD corresponding to the light and cleaved heavy chains, respectively (Figure 1).

Radiolabeling of Monoclonal Antibodies

Intact specific and control Mab or their $F(ab')_2$ fragments were labeled using chloramine-T method as follows. MAb MX-35 (1 mg) were added to 0.5 ml of 0.15M NaCl in 0.05M phosphate buffer at pH 7.5. One mCi of ^{125}I and 40μl of freshly dissolved chloramine-T (1 mg ml^{-1}) were added on ice. After 10 min the reaction was terminated by addition of 45 μl sodium metabisulfate (1 mg ml^{-1}). The protein was passed through a Sephadex G-25 column (10 ml) and fractions with the highest

radioactivity were pooled. The immunoreactivity of the labeled product was determined by sequential absorptions with an antigen-expressing cell line (OVCAR-3). Between 60 and 75% of the radioactivity was adsorbed for both $F(ab')_2$ fragments and intact antibody MX-35. Percent labeled protein was determined by the TCA precipitation method. For both $F(ab')_2$ fragments and intact antibody incorporation of iodine into protein was 90-95%.

Binding Assays

The method was described elsewhere (Mattes et al., 1989). Briefly, serial 2:3-fold dilutions of radiolabeled intact or $F(ab')_2$ fragments of MAb MX-35 were incubated with 50 μl cell suspension (5 μl packed OVCAR-3 cells) mixed with 100 μl of labeled MAb dilutions ranging from 0.05 $\mu g\ ml^{-1}$ to 20 $\mu g\ ml^{-1}$, for 5 hrs at 4 C. All assays were performed both with (2 mg ml^{-1}) and without a high concentration of unlabeled MAb MX-35. Free MAb was calculated by subtracting bound MAb from total bindable MAb, and the equilibrium association constant Ka was determined by graphical analysis using the method of Scatchard. The off-rates for radiolabeled MAb bound to OVCAR-3 cells were also determined as described by Mattes et al. (1989).

Biodistribution Studies

Athymic nude mice (BALB/c background) were implanted, intraperitoneally (IP), with fragments of the human ovarian cancer cell line, OVCAR-3, obtained by mincing tumor grown in another nude mouse. Visible tumors appeared approximately 3 weeks after injection. Each mouse had tumors of at least 300-500 mg. The expression of MX35 antigen in the tumor was confirmed by immunohistochemistry (Rubin et al. 1989). Mice bearing IP xenografts were randomized to be injected with either 20 μg of ^{125}I-labeled MAb MX-35 [intact or $F(ab')_2$] or the control antibody L6 anti-id 13B (intact or $F(ab')_2$) [kindly provided by Dr. I. Hellstrom, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA] either intraperitoneally or intravenously. The footnote to Table 1 summarizes the number of mice sacrificed at various time points for the eight possible combinations of treatment groups. Tumor tissues and normal tissues from each time point were obtained at the time of sacrifice by dissection. All tumor was recovered from sites within the peritoneal cavity. From

each animal 5 to 9 tumor samples were counted for ^{125}I. These animal experiments were performed in compliance with the relevant national laws relating to the conduct of animal experimentation.

Statistical Analysis

The uptake of antibodies was measured by calculating the % injected dose per gram of tissue. For tumor uptake and serum levels, mono-exponential models for the decay over time were fitted to the data using the method of analysis of covariance. The half-life of MAb serum levels and tumor were estimated using the regression coefficient

estimate B as T ½=log2 / (-B) (Gibaldi & Perrier, 1975).

Tumor specific localization was measured by the data obtained from tumor to blood, tumor to liver and tumor to muscle ratios of % injected dose. The effects of the type and the form of antibody, administration route and time were examined using the method of analysis of variance. Log transformations on the ratios were performed to ensure normality. The localization index (LI) (Beaumier et al., 1985) is defined as the ratio:

$$LI = \frac{\%\ \text{injected MX-35 g}^{-1}\ \text{tumor} \,/\, \%\ \text{injected control g}^{-1}\ \text{tumor}}{\%\ \text{injected MX-35 g}^{-1}\ \text{blood} \,/\, \%\ \text{injected control g}^{-1}\ \text{blood}}$$

A localization index of 1 means that the antibodies have similar standardized uptakes (Moshakis et al., 1981). An index greater than 1 indicates superior uptake of MAb MX-35 over control antibody.

In this study, the control and specific antibodies were not co-administered to the same animals but to two independent experimental groups. The mean tumor to blood ratio (in log scale) for each treatment combination was used to calculate LI. The standard errors of LI were calculated using assumption of normality on the logarithm of tumor to blood ratios.

Results

Characteristics of Radiolabeled Antibody and Fragments

The radiolabeling procedure did not significantly change the immunologic properties of both the whole IgG and $F(ab')_2$ fragments of MAb MX-35. They both retained 60-70% immunological reactivity after radiolabeling. The binding affinities of the intact and $F(ab')_2$ fragments of the antibody for OVCAR-3 cell line were determined by Scatchard analysis. Interestingly, the binding affinity of the intact MAb MX-35 (2.2×10^8 L M^{-1}) was slightly lower than that obtained with $F(ab')_2$ fragments (3.3×10^8 L M^{-1}). The number of binding sites/cell for the two forms was comparable (1.20×10^5 and 1.19×10^5 sites/cell, respectively). The off-rates for bound radiolabeled antibody were 23 hrs and 22 hrs for the intact antibody and fragment, respectively.

Pharmacokinetic Data

Serum clearance rates in 50 animals were determined for the ^{125}I-labeled intact MAb MX-35 and control antibody and in 58 animals for the ^{125}I-labeled $F(ab')_2$ fragments of MAb MX-35 and the control antibody for both IV and IP routes of administration (Table 1). The serum half-life was estimated as 31 hrs for IV and 39 hrs for IP injection for intact MAb MX-35 and 9 hrs for IV, 9.5 hrs for IP injection for the $F(ab')_2$ fragments of MAb MX-35. We could not demonstrate any statistically significant difference in the magnitude of antibody accumulation in tumors and serum between IV and IP routes of administrations for either intact or $F(ab')_2$ fragments of MAb MX-35 or control antibody (all $p > 0.07$) (Table 1). However, the difference between the specific MAb MX-35 and the control antibody L6 anti-id 13B and the difference

between intact and $F(ab')_2$ fragments were significant for serum clearance (Figure 2A) and tumor clearance (Figure 2B). A separate mono-exponential model was thus fitted to each of the four combinations. The models explained the data well as indicated by the correlation estimates. It is clear from Table 1 and Figure 2 that the clearance in serum is consistently faster than that from tumor. For both tumor and serum, the half-life of $F(ab')_2$ fragments are significantly shorter than that of intact MAb (all $p<0.01$). Also the half-life of intact MAb MX-35 was shorter than for control antibody in both tumor and serum (both $p<0.01$). However, the half-life of $F(ab')_2$ fragments of MAb MX-35 in tumor were longer than for the control antibody fragments ($P<0.01$) and in serum there were no differences between the half-lives of $F(ab')_2$ fragments of MAb MX-35 and the control antibody ($P<0.1$). As a result, the clearance of $F(ab')_2$ fragments of MAb MX-35 is relatively faster in serum than in tumor.

Biodistribution Data

Following injection of radiolabeled antibody, peak percent injected dose (mean value) per gram of tumor tissue was 1.6 at 24 hrs for IV and 2.4 at 12 hrs for IP routes of injection for the intact MAb MX-35 (Figure 2b). The corresponding values at 12 hrs were 10.4 for IV and 8.2 for IP administration for $F(ab')_2$ fragments of MAb MX-35. There was no specific localization of the control MAbs in the tumor with peak % injected dose per gram tissue of 0.4 and 2.0 for intact antibody and $F(ab')_2$ fragments, respectively both IV and IP routes of injection.

Accumulation of radioactivity in nomal tissues was consistently lower, for both intact mAb and fragments, than in tumor tissue. Table 2 gives some representative data for

one time point (24 hrs) and data for other time points are presented as tumor/normal tissue ratios in Table 3 and Figure 3. Ratios of tumor to blood, tumor to liver and tumor to muscle had similar patterns. The ratios of MAb MX-35 $F(ab')_2$ fragments increased with time and, in most cases reached a maximum at 61 hrs for both IV and IP routes of injection (Figures 3B, 3D, 3F). The ratios of intact MAb MX-35 also had an increasing trend when administered IV (Figures 3A, 3C, 3E). However, when given IP, neither intact MAb or $F(ab')_2$ fragments of the control antibody had apparent changes over time. The mean ratios at 61 hrs for $F(ab')_2$ fragments and the mean ratios at 72 hrs for intact antibody are tabulated in Table 3. This subset of data was chosen to be close to the peak area within the nature of the study design. Table 3 demonstrated that tumor to normal tissue ratios were much higher for MX-35 $(Fab')_2$ fragments than for any other combination and that the ratios for the IP and IV experimental groups were very similar.

Localization Data

Data for the localization of radiolabeled MAb in tumor relative to blood are shown in Figure 4. The mean LI for $F(ab')_2$ reached a value of 20.6 and 15.2 at 61 hrs for IV and IP injections, respectively, while mean LI for intact MAb was 4.8 and 4.3 for IV and IP injections, respectively with the former value being reached at 72 hrs and the latter at 24 hrs. The differences between IV and IP administrations were not significant except for that of intact antibody at 12 hrs ($p<0.05$) and 24 hrs ($p<0.02$). Generally, $F(ab')_2$ fragments had higher LI than that of intact antibody ($p<0.01$ at 48 hrs).

Discussion

Immunologic binding characteristics of the antibody and the accessibility of the antigenic sites in the tumor are the fundamental basis for the selection of MAbs for MAb-directed radio-immunodiagnosis or radioimmunotherapy of solid tumors. Monoclonal antibodies with desirable characteristics should produce high tumor uptake and accompanying low background activity, i.e., a high target to nontarget ratios, and uniform MAb accumulation within the tumor (Sands et al., 1990; Sharkey et al., 1990; Waldman et al., 1991). The delivery of MAbs into the tumor is influenced by two major determinants; 1) MAb properties such as binding affinity, size of the antibody molecule, dose, immunoreactivity and internalization, and 2) intrinsic tumor properties such as histology, antigen density and homogeneity, vascularity, blood flow, permeability, and size of the tumor (Jain, 1987). In our study, intrinsic tumor properties and MAb properties remained constant between the two experiments except for changes in molecular size of the MAb MX-35.

As the transport of solute molecules into the tumor interstitium is governed by the biological and physicochemical properties of the diffusing molecule, the molecular weight of the administered antibody will strongly influence the rate of passive diffusion across both interstitial and extravascular subcompartments and cell membranes (Jain, 1987). Inaccessibility of antigen-bearing tumor cells to MAbs appear to be a major cause of inhomogeneous distribution of MAb in the tumor. In order to circumvent the problems associated with poor penetration of MAb into tumors, molecules of lower molecular weight are preferable for *in vivo* immunotargeting. Also, an additional

advantage for low molecular-weight MAbs are their rapid clearance by urinary excretion (Bergardat et al., 1970; Buchegger et al., 1986; Endo et al., 1988). Moreover, the fragments lack the Fc region responsible for nonspecific tissue uptake. Radiolabeled fragments are, therefore, usually superior to whole IgG when used for *in vivo* immunotargeting (Buraggi et al., 1985; Andrew et al., 1986; Baum et al., 1986; Andrew et al., 1988). Although these advantages hold true for both Fab and $F(ab')_2$, reports dealing with studies on absolute tumor uptake of whole IgG, Fab and $F(ab')_2$ often show a decrease in tumor uptake of fragments when compared to whole IgG, most likely the result of increased clearance from the blood and a decreased affinity inherent in the generation of fragments (Buchegger et al., 1986; Colapinto et al., 1988; Endo et al., 1988); Wahl et al., 1983). Intact IgG usually gives the highest tumor uptake but this virtue may be overshadowed by high background levels of radioactivity. The renal clearance of whole IgG is relatively slow due to its high molecular weight, whereas fragments are rapidly cleared, thereby improving the tumor/background ratio.

In our investigation, we compared the characteristics of whole antibody MX-35 and its $F(ab')_2$ fragments with regard to biodistribution in nude mice bearing OVCAR-3 xenografts. We noted significant differences in biodistribution between labeled $F(ab')_2$ fragments and whole antibody MX-35, even though the immunoreactive fraction of the labeled antibody was very similar for both. Tumor to normal organ ratios were much higher for $F(ab')_2$ fragments as compared to IgG. Additionally, tumor to blood ratios for the $F(ab')_2$ fragments were approximately 7 times as high at 61 hrs as for IgG at

72 hrs or later (152 hrs) which is a similar finding to previous results obtained with intact MAbs and their fragments (Molthoff et al., 1992; Gerretsen et al., 1991). Interestingly, there was an approximately 5-fold increase in % injected dose per gram with $F(ab')_2$ fragments as compared to whole antibody resulting in a higher localization index (i.e., a higher specific/nonspecific tumor ratio). Our findings contrast with previous reports that have suggested that intact IgG's produce the highest levels of tumor uptake, while fragments produced significantly lower values. Molthoff et al. (1992) reported maximum absolute tumor uptake for intact IgG ranging from 8.5 to 17.7% injected dose per gram for antibody whereas for the respective $F(ab')_2$ fragments the maximum values were 5.2% to 10% injected dose per gram. In the same context, Gerretsen et al. (1992) reported a mean tumor uptake as 14% for whole IgG and 7.2% for $F(ab')_2$ fragments of the same antibody E48. However, in this particular study the investigators did not determine the binding affinities for the fragments. In a clinical study of mAb MOV18 in ovarian cancer patients, Buist et al. (1993) reported %ID/kg of 6.2 and 0.9 for intact IgG and $F(ab')_2$, respectively. The basis for the superior tumor uptake by fragments is in our study not clear. The effect, could be due to the slightly higher affinity shown by the fragments. It is also possible that the number of effective binding sites in the tumor is higher with $F(ab')_2$ fragments than with intact IgG due to better accessibility to the cell surface antigen as a consequence of reduced molecular size. Less surface area is occupied by the smaller $F(ab')_2$ molecules (Molthoff et al. 1992) which might escape a transportation barrier to which the intact antibody is vulnerable. Nevertheless, the number of

binding sites/cell measured *in vitro* with OVCAR-3 cells was very similar for both forms of the MAb MX-35. Also, the off-rates for the cell-bound whole IgG and the fragment are very similar. Another possibility is that the intact MAb may be reacting with Fc receptors in tissues or in blood cells, although this is unlikely as mouse IgG, antibodies such as MAb MX-35, do not show this property. The explanation for the superior properties of the fragment will be the subject of further studies.

In summary, the use of $F(ab')_2$ fragments of MAb MX-35 strongly improved absolute tumor uptake of the MAb when compared directly with intact MAb MX-35. An on-going clinical study in patients with epithelial ovarian cancer on the localization of ^{125}I-labeled whole MX-35 antibody has demonstrated modest accumulation (mean %ID g^{-1} = 1.08×10^{-3} at 7 days) of the antibody in tumors (Rubin et al., 1993). These data are comparable to the values noted in the present animal study for whole antibody. This study therefore provides a rationale for a clinical study in patients with epithelial ovarian cancer patients using radiolabeled $F(ab')_2$ fragments.

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Table 1. **Comparison of Serum and Tumor Half Lives of Whole Antibody and Antibody Fragments**

		Antibody half-life (hours)	
MAb[a]	Route	Serum	Tumor
MX-35 intact	IV	30.8	42.0
	IP	39.3	36.7
L6[c] intact	IV	ND[b]	ND
	IP	77.5	141.6
MX-35 $F(ab')_2$	IV	9.0	16.6
	IP	9.5	16.6
L6[c] $F(ab')_2$	IV	10.9	12.0
	IP	10.1	12.1

[a] For intact and $F(ab')_2$ forms of MX-35, 3 or 4 mice were used for each time point (IV or IP) except for the 61 and 68 hr time points with the $F(ab')_2$ fragments when 2 mice were used. For the control MAb, 2 mice were used for each time point except for the 24, 61 and 68 hr IP points when one mouse was used.

[b] Not determined. The antibody level essentially did not decline during this time period. The slope was close to 0.

[c] MAb L6 anti-id 13B was used as control.

Table 3. **Summary of Biodistribution Data of the Radiolabeled Antibodies**

Monoclonal Antibody	Route	Ratios		
		Tumor:Blood	Tumor:Muscle	Tumor:Liver
MX-35 intact	IV	1.9	12.3	6.3
	IP	1.4	9.0	3.7
L6[c] intact	IV	0.4	2.8	1.6
	IP	0.5	2.9	2.0
MX-35 $F(ab')_2$	IV	12.9	49.2	9.3
	IP	11.5	64.2	28.4
L6[c] $F(ab')_2$	IV	0.6	5.0	2.0
	IP	0.8	5.1	2.0

All of the above means were taken in log scale. The mean ratios are tabulated for intact antibody at 72 hrs and for $F(ab')_2$ fragments at 61 hrs. See Table 1 for number of mice used in each experimental group.

[c] MAb L6 anti-id 13B was used as control.

Brief Description of the Figures

Figure 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis under reducing conditions of MAb MX-35 (lanes a, b, c) and control MAb L6 anti-id 13B (lanes d, e, f) before enzyme treatment (lanes a, d); after pepsin digestion overnight at 37 C (lanes b, e); and following passage through a protein A column to remove undigested intact IgG (lanes c, f). The gel bands are visualized by Coomassie blue staining. Bands of Mr 50,000, Mr 25,000 and Mr 23,000 correspond to the heavy chains (hc), heavy chain fragments (hc-f), and light chains (lc), respectively.

Figure 2: Mean percentages of injected radioactivity in blood (Panel A) and in tumor (Panel B) showing clearance of intact IgG and $F(ab')_2$ fragments of MAb MX-35 and control MAb L6 anti-id 13B. For intact IgG N=6 for all time points. For $F(ab')_2$ fragments of MAb MX-35 N=7 at 12, 24, and 48 hours, N=8 at 38 hours and N=5 at 61 and 68 hours. The experimental groups (IP and IV combined) are distinguished as follows: intact MAb MX-35 (black dashed line); MAb MX-35 $F(ab')_2$ fragments (black solid line); control intact MAb L6 (stippled dashed line); and control MAb L6 $F(ab')_2$ fragments (stippled solid line). Vertical bars indicate range or +/-2 standard errors.

Figure 3: Biodistribution data indicating tumor to blood ratios (A, B), tumor to muscle ratios (C, D); and tumor to liver ratios (E, F) and comparing intact IgG (A, C, E) and $F(ab')_2$ fragments (B, D, F). All groups

consisted of 3 or 4 mice per time point. The ratios for $F(ab')_2$ fragments continued to increase up to 68 hours whereas intact antibody did not show an apparent change over time. The experimental groups are distinguished as follows: intact or fragmented MAb MX-35 by IP injection (black solid line) or by IV injection (black dashed line); intact or fragmented control MAb L6 by IP injection (stippled solid line) or by IV injection (stippled dashed line). Vertical bars indicate range or +/- 2 standard errors.

Figure 4: Comparison of tumor localization for intact IgG and $F(ab')_2$ fragments. The localization index reflects the ratio of activities in tumor of "specific" intact MAb MX-35 and $F(ab')_2$ fragments over "nonspecific" control intact MAb L6 and $F(ab')_2$ fragments and corrected for blood activity at the same time. There was much more rapid development of specific uptake with $F(ab')_2$ fragments of MAb MX-35 as compared to the intact MAb MX-35. The experimental groups are distinguished as follows: intact IgG by IP injection (stippled dashed line) or by IV injection (stippled solid line); $F(ab')_2$ fragments by IP injection (black dashed line) or by IV injection (black solid line). Vertical bars indicate range or +/-2 standard errors.

Synopsis:

This is a Phase I dose escalation study using ^{131}I-MX35 F(ab')$_2$ (2 mg/dose) for treatment of patients with low volume (≤ 0.5 cm diameter, documented at post-chemotherapy laparotomy) MX35 antigen-expressing (≥ 25% of cells in an average high power field) ovarian carcinoma. Three patients per dose level will be followed for up to 8 weeks with biochemical and hematologic tests for toxicity, and CA-125 estimations for response. The first cohort of patients will receive 30 mCi ^{131}I labeled to 2 mg MX35 F(ab')$_2$ antibody. Subsequent dose escalation will be in 30 mCi ^{131}I (labeled to 2 mg MX35 F(ab')$_2$). Patients will be treated as outpatients in the Adult Day Hospital. Doses will be administered at intervals of 4-5 days. Patients at the first dose level will receive one outpatient infusion. Patients at subsequent dose levels will receive repeat outpatient infusions at least 4 days after the prior infusion, with total patient radiation levels to be ≤ 5 mR/h at 1 meter at completion of infusion. All infusions will be completed within 14 days, to obviate/minimize anti-murine antibody responses *during* infusion. In the absence of disease progression and after recovery from toxicity patients may be re-treated beginning 8 weeks after the prior infusion, for a total of not more than three treatments.

A Maximum Tolerated Dose (MTD) will be determined, defined as the highest dose at which not more than a third of the patients have Grade IV hematopoietic or Grade III or greater non-hematopoietic toxicity. Serologically evaluable patients will be studied for possible responses.

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